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THE EFFECT OF 2,4-DINITROPHENOL ON THE TISSUE CONCENTRATION OF IODINE-CONTAINING COMPOUNDS IN ISOTOPICALLY EQUILIBRATED INTACT RATS

BY GABRIELLA MORREALE DE ESCOBAR
AND F. ESCOBAR DEL REY

From the Instituto 'G. Marañón', Consejo Superior de Investigaciones Científicas, Centro de Investigaciones Biológicas, Velázquez, 138-Madrid, Spain

(*Received* 25 *January* 1961)

It has been repeatedly shown (Wolff, Rubin & Chaikoff, 1950; Goldberg & Chaikoff, 1951; Goldberg, Wolff & Greep, 1955, 1957; Castor & Beierwaltes, 1956) that the administration of 2,4-dinitrophenol to mammals is accompanied by a sharp decrease of the plasma PBI without an increase of TSH secretion by the pituitary. (The following abbreviations are used: 2,4-dinitrophenol (DNP); thyroid-stimulating hormone (TSH); protein-bound iodine (PBI); trichloracetic acid precipitable (TCAp); thyroxine-binding globulin (TBG).) These findings are usually considered to be due to 'disruption' of the classical thyroid–pituitary feed-back mechanism. Goldberg *et al.* (1957) showed that the pituitaries of DNP-treated animals had not lost the ability of reacting to other stimuli, and concluded from their results that the data 'were not consistent with the simple classical feed-back theory of thyroid–pituitary interrelationships. It is likely that the pituitary cells secreting TSH are influenced not only by the actual circulating level of thyroid hormone(s) but by one or more of its peripheral actions'.

Data obtained while studying the influence of DNP on the peripheral metabolism of L-thyroxine in thyroidectomized, L-thyroxine-maintained rats (Escobar del Rey & Morreale de Escobar, 1958*a*, *b*) led us to suggest that the sharply lowered plasma PBI of treated animals might not necessarily mean that a similar decrease had occurred in the concentration of thyroid hormone in the peripheral tissues: we had observed and have later confirmed in more extensive experiments *in vivo* and *in vitro* (Morreale de Escobar & Escobar del Rey, 1961) that DNP alters the normal partition of the thyroid hormone between plasma or protein-free incubation media and cells in favour of the latter.

It has been the working hypothesis for this work that, in non-thyroidectomized rats receiving DNP, the tissue concentration of thyroid hormone(s)

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It has been repeatedly shown (Wolff, Rubin & Chaikoff, 1950; Goldberg & Chaikoff, 1951; Goldberg, Wolff & Greep, 1955, 1957; Castor & Beierwaltes, 1956) that the administration of 2,4-dinitrophenol to mammals is accompanied by a sharp decrease of the plasma PBI without an increase of TSH secretion by the pituitary. (The following abbreviations are used: 2,4-dinitrophenol (DNP); thyroid-stimulating hormone (TSH); protein-bound iodine (PBI); trichloracetic acid precipitable (TCAp); thyroxine-binding globulin (TBG).) These findings are usually considered to be due to 'disruption' of the classical thyroid–pituitary feed-back mechanism. Goldberg *et al.* (1957) showed that the pituitaries of DNP-treated animals had not lost the ability of reacting to other stimuli, and concluded from their results that the data 'were not consistent with the simple classical feed-back theory of thyroid–pituitary interrelationships. It is likely that the pituitary cells secreting TSH are influenced not only by the actual circulating level of thyroid hormone(s) but by one or more of its peripheral actions'.

Data obtained while studying the influence of DNP on the peripheral metabolism of L-thyroxine in thyroidectomized, L-thyroxine-maintained rats (Escobar del Rey & Morreale de Escobar, 1958*a*, *b*) led us to suggest that the sharply lowered plasma PBI of treated animals might not necessarily mean that a similar decrease had occurred in the concentration of thyroid hormone in the peripheral tissues: we had observed and have later confirmed in more extensive experiments *in vivo* and *in vitro* (Morreale de Escobar & Escobar del Rey, 1961) that DNP alters the normal partition of the thyroid hormone between plasma or protein-free incubation media and cells in favour of the latter.

It has been the working hypothesis for this work that, in non-thyroidectomized rats receiving DNP, the tissue concentration of thyroid hormone(s)

might remain within normal limits, despite the very low plasma level. If this were confirmed, the lack of a TSH-secreting response to the sharp lowering of the plasma PBI might be interpreted as an indication that the mechanisms controlling TSH secretion are more sensitive to factors related to the intracellular concentration of thyroid hormone than to its circulating level. Whenever the plasma concentration of thyroid hormone no longer reflected intracellular events, PBI data alone might lead us to conclude wrongly that there was 'disruption' of the thyroid–pituitary servo mechanism.

The concentration of iodinated compounds in tissues was taken as an index of their thyroid hormone content. Owing to difficulties inherent in present techniques for the chemical determination of iodinated compounds in most tissues, an isotope equilibrium dilution method was adopted, similar to that proposed by van Middlesworth (1956).

METHODS

Unless otherwise stated the general procedure was as follows: either male or female young adult Wistar rats were fed on a low-iodine, medium-residue, Remington diet for at least a month before and during equilibration with radio-iodide. Since its exact iodine content was not known, the same batch of feed was used for a given group throughout the experiment.

Isotopic equilibration of the iodine-containing compounds in the animals was achieved by the daily administration (in the feed or by intraperitoneal injection) of radio-iodide, following in general the procedure of van Middlesworth (1956). The dose range for different experimental groups was I^- 2–4 μg/rat/day, labelled with less than 1 μc of ^{131}I, as determined on the first day of administration. Attainment of isotopic equilibrium was assessed for each experimental group by one or more of the following criteria: attaining a plateau of neck ^{131}I count and of the individual urinary and faecal excretions; recovery of at least 95 % of the daily administered dose in the excreta; constancy of plasma, tissue and thyroid ^{131}I; constancy of the specific activity of the plasma PBI. By these criteria and in these experimental conditions, isotopic equilibration of the major iodine compartments was reached in 16–20 days.

Half the equilibrated group then received 2–4 mg DNP/rat/8 hr in the feed or intraperitoneally. These animals and their untreated controls were killed at different times after the last DNP injection. Blood was withdrawn under ether narcosis after I.V. heparin, and the animals were then exsanguinated from the inferior vena cava. It later became necessary to alter the procedure by perfusing the animals at necropsy with 150 ml. of saline, after extraction of about 5 ml. of blood. The technique was checked in a group of radio-iodide-equilibrated rats, injected with Evans Blue and killed with perfusion half an hour later; it was then found that the ratio of colour to TCAp-^{131}I was the same in both whole plasma and perfusate. The ^{131}I distribution throughout the whole body was determined as previously described (Escobar del Rey & Morreale de Escobar, 1958*a*); the procedure for the determination of the TCA-precipitable fraction of iodinated compounds was, however, modified by the addition before fractionation of thiourea and of non-radioactive plasma (the latter to tissue samples) in order to avoid spurious results due to the presence of haemoglobin (Rosenberg, 1959) and to ensure a sufficient thyroxine-binding protein content.

Radioactivity was determined with a thallium-activated sodium iodide well-type scintillation crystal and the results are expressed as percentage of the dose of radio-iodide administered daily. Since the animals were maintained in isotopic equilibrium until necropsy,

it is valid to assume that the concentration of iodinated compounds in a given sample was proportional to its radioactivity. The minimum iodine content in a given sample may be calculated from the latter and from the specific activity of the exogenous iodide. External sources of iodine other than the administered labelled iodide were kept as low and as constant as possible, and should influence figures of both DNP-treated and untreated animals in the same way. Since for technical reasons the total iodine intake was not known, the results reported here are expressed as percentages of the known dose administered daily and not in mass units.

Stable iodine determinations in plasma were carried out by the wet-ashing procedure of Connor, Swenson, Park, Gangloff, Lieberman & Curtis (1949).

Carrier-free ^{131}I-iodide was obtained from the Radiochemical Centre, Amersham (England). L-Thyroxine, labelled in the 3′- and 5′-positions was supplied by Abbott Laboratories (U.S.A.).

DNP was purified by two crystallizations from acetone; it was dissolved in NaOH solution and neutralized with HCl so that the final solution was approximately 0·9 g/100 ml. NaCl solution. When DNP was injected intraperitoneally, the dose was contained in 0·5 ml.; equal volumes of isotonic saline were injected into the controls.

RESULTS

Experiments with several different groups of animals as described above repeatedly showed both with males and females (Table 1) that the ratio between the TCA-precipitable iodinated compounds in the carcass to that in the plasma was always higher in the DNP-treated animals, the increase being 33–200% of the control value. (By 'carcass' is understood the mechanical homogenate consisting mostly of skeletal muscle and bones obtained after removal of blood, organs, gastro-intestinal tract, abdominal fat and skin.) Though not shown in this table, the same occurred for the ratio between heart, brain, kidney, spleen, etc., and plasma. This 'shift' in

TABLE 1. The effect of DNP administration on the tissue-plasma partition of iodine-containing compounds in rats isotopically equilibrated with radio-iodide

		$\frac{\text{TCAp } ^{131}\text{I in 1 g carcass}}{\text{TCAp } ^{131}\text{I in 1 ml. plasma}}$			
Group	Time on DNP	Control	DNP-treated	*P*	Increase over control value (%)
I *f*	24 hr	0·23	0·39	< 0·001	70
	5 days	0·23	0·42	< 0·001	83
II *f*	22 hr	0·21	0·28	< 0·01	33
	5 days	0·25	0·35	< 0·02	40
III *m*	5 days	0·10	0·16	< 0·001	58
IV *f*	4 days	0·20	0·60	< 0·001	200
*V *m*	5 days	0·10	0·25	< 0·001	150
VI *m*	4 days	0·15	0·40	< 0·001	167

Animals of all these groups were in isotopic equilibrium with exogenous labelled iodide as ascertained by several criteria (see Methods); other experimental conditions, such as radio-iodide dose and mode of administration, DNP dose level, route and frequency of administration, moment of observation of ^{131}I distribution, were different.

* Underlined groups correspond to animals thoroughly perfused at necropsy.

the distribution of iodine-containing compounds between plasma and tissues in favour of the latter resulted from the sharp decrease during DNP administration of the plasma concentration (down to 50–25 % of the control value), whereas the concentration of such compounds in the carcass remained unaltered in most experimental groups or decreased somewhat in others, but always to a lesser extent than did the plasma concentration.

TABLE 2. Influence of DNP on the distribution of iodine-containing compounds in rats isotopically equilibrated with radio-iodide

	% of daily dose: mean ± S.D.		
	DNP-treated (4)	Control (4)	*P*
Thyroid	424 ± 59	411 ± 63	—
Plasma (1 ml.)	0·285 ± 0·026	0·942 ± 0·084	< 0·001
Plasma (1 ml.) TCAp	0·202 ± 0·025	0·840 ± 0·100	< 0·001
Carcass (1 g)	0·184 ± 0·028	0·187 ± 0·013	—
Carcass (total)	19·5 ± 2·7	16·9 ± 2·3	—
Liver (total)	4·03 ± 1·08	6·48 ± 1·73	—
Intestines (total)	11·61 ± 3·40	8·48 ± 2·55	—
Carcass: plasma ratio	0·66 ± 0·18	0·18 ± 0·5	0·01–0·001

Female rats, weighing 180–200 g, isotopically equilibrated with radio-iodide 4 μg/rat/day for 25 days, treated for 5 days with 3 mg DNP/8 hr; killed 4 hr after the last injection of the drug.

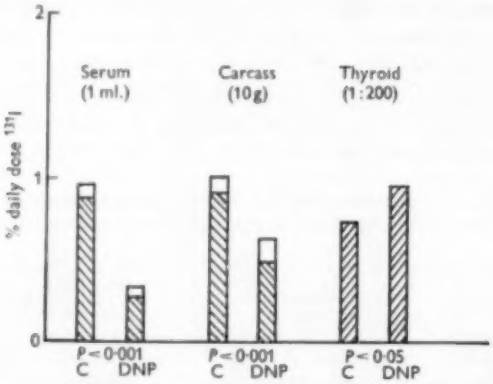

Fig. 1. Influence of DNP on the content of iodine-containing compounds in plasma, carcass and thyroid. Male rats, weighing 240–260 g, isotopically equilibrated with radio-iodide 3 μg/rat/day for 5 days and killed 7 hr after the last injection. The means of data from ten animals in each group are indicated. ▨ TCAp ^{131}I; □ TCA soluble ^{131}I; ▨ total ^{131}I.

In those experimental groups where the carcass radioactivity remained the same in both DNP-treated and control animals, no change was observed in the mean thyroid ^{131}I content. On the contrary, in some animals where a decrease of the carcass concentration of iodine-containing compounds had occurred, an increase of the mean total thyroid ^{131}I was found.

Table 2 and Fig. 1 show typical examples of both types of response. This difference in response to the administration of DNP from one experimental group to another was most unsatisfactory; similar variability in the response to DNP of other parameters had also been observed by others (Goldberg & Chaikoff, 1951; Goldberg *et al.* 1957). It did not appear to depend on the sex of the animals used. Closer examination of the experimental conditions employed and the type of response obtained showed that whenever a decrease in the concentration of iodine-containing compounds had been observed in the carcass, the animals had been killed 6–8 hr or more after the last dose of DNP. Indeed, the lowest tissue concentrations we observed were found in an experimental group which had been killed 12 hr after the administration of the daily feed containing 12 mg of the drug. The highest tissue concentrations of iodine-containing compounds was found in animals killed about 4 hr after the last DNP injection. On the other hand, *in vitro* experiments which were being carried out simultaneously (Morreale de Escobar & Escobar del Rey, 1961) showed that the increase induced by DNP in 'uptake' of ^{131}I-L-thyroxine by rat R.B.C. and diaphragms depended on the concentration of the latter and needed time to reach a maximum. It was therefore possible that, as time elapsed after the administration of DNP to the animals, the circulating concentration of the drug decreased, and with it the extent of the 'shift' of iodine-containing compounds from the plasma to tissues.

The effect of DNP on the tissue concentration of iodine-containing compounds was therefore re-investigated at different times after DNP administration; some of the results obtained for a group of female rats weighing 180–200 g at necropsy, isotopically equilibrated on 4 μg I^-/rat/day for 25 days, which had received 3 mg DNP every 8 hr during the last 4 days, are shown in Fig. 2. As may be seen, the concentration of iodinated TCA-precipitable compounds in the plasma of the DNP-treated animals was about 25% that of the controls during the whole period of observation. On the other hand, the concentration of these compounds in the carcass (and, though not shown in the figure, in brain, heart, etc.) became the same as that of the control group 1 hr after the last intraperitoneal injection of DNP and was still so at 4 hr. By 8 hr it had decreased to about two-thirds of the control value. The carcass:plasma ratio of iodine-containing compounds clearly reflected these findings, showing a rapid increase soon after the injection of DNP and reverting almost to control values by 8 hr, at which time the concentration of DNP in the plasma had fallen to low levels. The thyroid iodine remained unaltered in both groups during the whole period. Since the animals had been receiving the drug periodically (every 8 hr) it seems valid to conclude that a similar pattern of events as that found during the 8 hr observation period (Fig. 2) had been

repeating itself, the plasma concentration of iodinated compounds being continuously low, that in the tissues varying within limits much nearer to the control range.

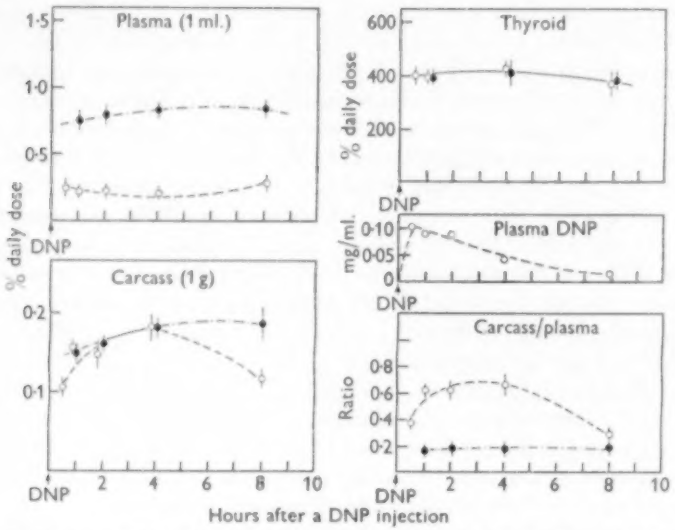

Fig. 2. Influence of DNP on the distribution of iodine-containing compounds in the plasma, carcass and thyroid at different times after the last injection of the drug. Female rats, weighing 180–200 g, isotopically equilibrated with 4 μg radio-iodide/rat/day over a 25-day period, treated with 3 mg DNP/8 hr for 4 days. Data are expressed as percentage of the administered radio-iodide; the mean $\pm$ S.D. being given. Changes in the plasma concentration of DNP with time are also shown. ○ DNP-treated; ● controls.

From other experiments another factor which might have been influencing these results emerged. In order to determine the ratio of carcass to plasma concentrations of iodine-containing compounds for very low and very high levels of circulating thyroid hormone, thyroidectomized rats were maintained and isotopically equilibrated at different dose levels of L-thryoxine (0·4–100 μg L-T_4/rat/day). It was found that over a very wide concentration range there was roughly ten times more TCA-precipitable iodine in 1 ml. plasma than in 1 g peripheral muscle, heart, brain, etc. This would mean that blood retained in the tissues would give artificially high values of the tissue concentrations of iodinated compounds. It was therefore possible that at least part of the decrease in iodinated compounds that had been observed in the peripheral muscle of some DNP-treated groups was actually due to the great decrease in the concentration of these

compounds in the plasma retained in tissues at necropsy. Assuming the true (no retained plasma) concentration of iodine-containing compounds of the peripheral muscle of DNP-treated and control animals to be the same, the plasma content for DNP-treated rats to be one-third that of controls, and that about 3 ml. of plasma could easily remain in 100 g of peripheral muscle at necropsy, a 20 % decrease of the apparent carcass concentration in DNP-treated animals would be accounted for. A greater decrease in the plasma concentration would result in an even larger artifactual difference between the carcass data for DNP-treated and control animals.

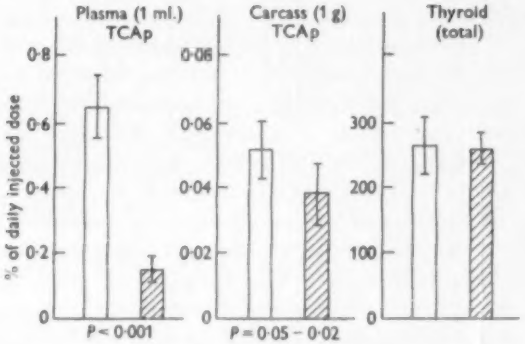

Fig. 3. Plasma, peripheral muscle and thyroid content of iodinated compounds in male rats, weighing 260–280 g, isotopically equilibrated on radio-iodide 4 μg/rat/day over a 26-day period, treated with 10 mg DNP in the feed for 4 days and killed with thorough perfusion. The mean $\pm$ S.D. is indicated. ▨ DNP-treated; □ control.

For this reason the experimental procedure was altered to include thorough perfusion of the rats at necropsy. Figure 3 shows the results obtained in such an experiment with a group of male rats, weighing 260–280 g, which had been isotopically equilibrated with 4 μg I^-/rat/day and had been treated for 4 days with 10 mg DNP in the feed and 3 mg by intraperitoneal injection 20 and 3 hr before being killed. As may be seen, the plasma concentration of TCA-precipitable iodine-containing compounds was very low in the DNP-treated animals, being about one-fourth that of the controls; the concentration in the perfused carcass decreased about 20 %, the difference between both mean values being near the borderline of statistical significance (P = 0·05–0·02). The carcass:plasma ratio was therefore much higher for the DNP-treated animals. Total thyroid iodine content remained unchanged in both groups.

When the changes with time in the distribution of iodine-containing compounds were determined in groups of male rats, weighing 210–250 g, isotopically equilibrated on 4 μg I^-/rat/day, treated during 5 days before

necropsy with 3 mg DNP/8 hr, and killed with thorough perfusion at intervals up to 11 hr after the last DNP injection, it was found (Fig. 4) that the plasma concentration of iodine-containing compounds in DNP-treated animals was about one-third the control value, whereas the carcass concentration was the same as that in control animals in some groups, higher or lower in others, the over-all mean being the same. As has already

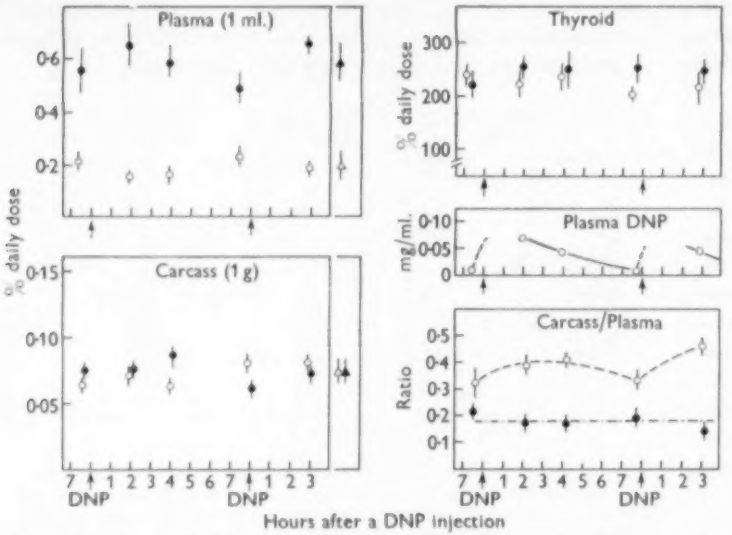

Fig. 4. Variations with time of the effect of DNP on the concentration of iodine-containing compounds in the plasma, peripheral muscle and thyroid of male rats, weighing 220–250 g, isotopically equilibrated on radio-iodide 4 μg/rat/day, treated for 4 days with 3 mg DNP/8 hr and killed with thorough perfusion: mean $\pm$ S.D. of groups of four animals are given. The variations with time of the plasma DNP concentration are also shown. ○ DNP-treated; ● controls; △, ▲ over-all means $\pm$ S.D. over whole period of observation.

been seen in other experiments (Table 1 and Fig. 2), a 'shift' of iodine-containing compounds from the plasma to tissues occurred in DNP-treated animals and this 'shift' was least when the plasma DNP concentration was lowest. The mean values for the thyroid iodine content were the same for both treated and untreated rats. However, when the individual thyroidal iodine contents were plotted against the concentration of iodine-containing compounds in the carcass for animals of both groups together, a negative correlation was found ($P < 0{\cdot}05$); for DNP-treated rats alone, the thyroid–carcass correlation was significant at a $P = 0{\cdot}01$–$0{\cdot}001$ level. No correlation was found when the thyroid values were plotted against corresponding plasma values.

As has been stated above and shown in Table 2 and Fig. 2, the apparent carcass concentrations of iodine-containing compounds in many DNP-treated groups was the same as that in the corresponding controls. From the results obtained with perfused animals (Fig. 4) it would not seem too speculative to assume that, in some at least of the unperfused groups, the true tissue concentration of iodine-containing compounds in DNP-treated animals had actually been higher than in the controls and that some of the differences between the carcass data for both groups had been smaller.

The present results underline the necessity of determining the pattern of distribution of iodine-containing compounds at different times during the experimental procedure, since data obtained at one time only might be misleading as to the general sequence of events (see Figs. 3 and 4). Moreover, we think that changes in dose level, mode and frequency of administration of DNP might lead to quantitatively different patterns of distribution of iodinated compounds in plasma and tissues; this is a possible explanation for many of the differences in the response to DNP described by others (Goldberg & Chaikoff, 1951; Goldberg *et al.* 1957). Total perfusion of the animals to eliminate plasma from the tissues is also a necessity if data on the iodine content of the latter are sought.

Though the data are not shown here in detail, the concentration of iodinated compounds in the heart was usually lower in the DNP-treated animals than in their controls, even in those perfused groups where no difference was demonstrable in peripheral muscle. The concentrations in the brain, on the contrary, behaved similarly to that in the corresponding peripheral muscle in all groups.

We thought it would be interesting to determine the concentration of iodine-containing compounds in the pituitaries of DNP-treated and control, radio-iodide-equilibrated, intact rats; this was not feasible under our experimental conditions, because of the extremely low radioactivities encountered in the glands. For this reason measurements were attempted in thyroidectomized male rats weighing 200–250 g, isotopically equilibrated on ^{131}I-L-thyroxine 4 μg/rat/day. In such experimental conditions isotopic equilibrium is achieved in a much shorter period (5–6 days) and without concentration of radioactivity in a small gland, thus permitting the use of higher initial radioactivities (about 3·5 μc/4 μg L-T_4 as determined on the first day of administration). Preliminary results with perfused animals show that the iodine content of total pituitaries of DNP-treated animals was the same as that of the controls (0·0048 $\pm$ 0·0007 % dose for the former and 0·0050 $\pm$ 0·0008 for the latter), despite the sharp decrease of the concentration of iodine-containing compounds in the plasma (1·37 $\pm$ 0·17% dose for DNP-treated versus 3·06 $\pm$ 0·21 for the untreated control). The corresponding data for the brain were 0·37 $\pm$ 0·04 % and 0·38 $\pm$ 0·03 %.

Moreover, the variations with time of the iodine content of the pituitaries parallelled the changes occurring in peripheral muscle and brain more closely than those taking place in the plasma.

Because of the low radioactivities of the samples obtained from radioiodide-equilibrated intact rats a reliable chromatographic identification of the nature of the iodinated compounds in the plasma and tissues was not feasible, without using dangerously high initial radioactivities.

DISCUSSION

From the experiments reported here we have concluded that in DNP-treated rats the lowering of the concentration of TCA-precipitable iodine-containing compounds in the plasma does not reflect changes in the concentration of these compounds in peripheral tissues, such as skeletal muscle, brain, etc. Indeed, the over-all concentration of such compounds in these tissues remains within a range comparable to that in untreated rats, despite the continuously low circulating level. Preliminary data suggest that variations in the iodine concentration in pituitaries of DNP-treated rats reflect alterations occurring in brain and skeletal muscle more closely than those taking place in the plasma.

Such a pattern of distribution of iodine-containing compounds appears to be brought about chiefly by two factors: (*a*) The very rapid decrease in the plasma level of iodinated compounds was shown (Escobar del Rey & Morreale de Escobar, 1958*a*, *b*) to be accounted for by the simultaneous intense increase of their biliary secretion and, ultimately, a faecal excretion. (*b*) The maintenance of a normal or nearly normal concentration of iodine-containing compounds in most peripheral tissues probably results from the alteration induced by DNP in the normal partition of thyroid hormone between plasma and tissues in favour of the latter. This effect is reflected in the variations of the carcass-to-plasma ratios of TCA-precipitable iodine-containing compounds reported here. It is also suggested by the increased red-blood-cell radioactivity in animals injected with labelled thyroxine and DNP (Escobar del Rey & Morreale de Escobar, 1958*b*). Moreover, *in vitro* determinations of the influence of DNP on the 'uptake' of thyroxine by human and rat red blood cells and by diaphragms of perfused rats consistently showed (Morreale de Escobar & Escobar del Rey, 1961) that the drug greatly increases the proportion of available hormone which is found with the red-blood-cell fraction even under experimental conditions which exclude a mechanism involving solely and/or principally an action on the plasma-protein–hormone complex invoked by others (Christensen, 1959, 1960). It was concluded that this DNP effect is chiefly exerted directly on the cells, the exact site remaining undetermined.

It is not known whether the increased biliary secretion of thyroid hor-

mone, which is responsible for the low plasma PBI (Escobar del Rey & Morreale de Escobar, 1958*b*) is also the result of an increased transfer of thyroxine to the liver. If this were so, the entire distribution pattern of iodine-containing compounds in these DNP-treated animals could be accounted for by the 'shift' of the hormone towards the cells.

The situation during DNP administration in the rat may be summarized as follows:

(*a*) There is no increase in the TSH-secreting activity of the pituitary (Goldberg & Chaikoff, 1951; Goldberg *et al.* 1955, 1957).

(*b*) As is shown here, the concentration of iodine-containing compounds in peripheral tissues, pituitary included, remains within normal limits; the over-all peripheral deiodination of L-thyroxine is also unaltered or somewhat increased (Escobar del Rey & Morreale de Escobar, 1958*a*, *b*).

(*c*) It has been repeatedly shown (Wolff *et al.* 1950; Goldberg & Chaikoff, 1951; Goldberg *et al.* 1955, 1957; Escobar del Rey & Morreale de Escobar, 1958*a*, *b*), and is confirmed here, that there is a very sharp and rapid decrease of the plasma PBI.

(*d*) DNP alters both *in vivo* and *in vitro* the distribution of thyroid hormone between plasma and tissues in favour of the latter; data reported elsewhere (Morreale de Escobar & Escobar del Rey, 1961) support the conclusion that this effect of DNP is mainly exerted by a direct action on the cells.

(*e*) A negative correlation between the content of iodine-containing compounds of the thyroid and that of the peripheral muscle was sometimes observed, but none between the thyroid content and the plasma PBI.

These points have led us to the following tentative conclusions: The level of circulating thyroid hormone is not always a good index of its concentration and metabolism in the peripheral tissues. The same might be said of the level of 'free' circulating thyroxine. The behaviour of the TSH-secreting cells of DNP-treated rats would seem to correlate better with parameters such as the concentration and disposal of thyroid hormone in the peripheral tissues than with its level, whether total or 'free', in the circulation.

Though evidence is still fragmentary, there is general agreement that thyroid-hormone–plasma-protein interactions may control the action of the thyroid hormone by determining the level of its 'free' diffusible form in the circulation and hence its transfer to the peripheral tissues (Robbins & Rall, 1957, 1960; Pitt-Rivers & Tata, 1959; Dowling, Freinkel & Ingbar, 1960). The concentration of free diffusible thyroxine in the blood would therefore play a major role in the thyroid–pituitary homoeostatic

mechanism, a system which is believed to be regulated so as to maintain a normal intracellular concentration and metabolism of the thyroid hormone. We would point out that in such a scheme the level of free thyroid hormone in the circulation would be assigned a primary role in the regulation of TSH secretion only in so far as it determined the rate of transfer and of disposal of the hormone in the tissues. However, it appears from the present data and those reported elsewhere (Morreale de Escobar & Escobar del Rey, 1961) that these parameters might be influenced directly via mechanisms not operating at the level of the plasma proteins, but of the cells. We venture to suggest as a working hypothesis that TSH-secreting cells are sensitive to parameters related to the metabolic situation of the thyroid hormone in the peripheral tissues. That this might occur at a hypophysial level would not seem excluded by the present results nor by those reported by others (von Euler & Holmgren, 1956*a*, *b*; Yamada, 1959*a*, *b*). Whenever the concentration of circulating thyroid hormone no longer reflected these tissue parameters, as appears to occur in DNP-treated rats, if the former alone were considered, the situation would be one of an apparent 'disruption' of the thyroid–pituitary servo mechanisms.

These remarks are not meant to convey that the regulating role in thyroid–pituitary interrelationships should be assigned to the tissue thyroid hormone concentration *per se*, but to factor(s) somehow related to it. Too little is known at present about possible interrelationships, if any, between intracellular concentration, degradation, metabolic action, etc., of the hormone to venture a more precise hypothesis.

It seems, therefore, desirable whenever possible to obtain quantitative data on the concentration and metabolism of iodinated compounds in the peripheral tissues, specially at a hypophysial and hypothalamic level. Such information might prove quite valuable in giving us a deeper insight into the regulators more directly involved in thyroid–pituitary interplay and the factors which might modify them.

SUMMARY

1. The effect of the administration of 2,4-dinitrophenol for several days on the concentration of iodine-containing compounds in plasma and tissues of rats was determined, using animals isotopically equilibrated with radio-iodide.

2. It was found that the concentration of TCA-precipitable iodine-containing compounds in the plasma of DNP-treated animals was one-third or less that of their controls.

3. The interval between the last administration of DNP and the observations influences the actual concentration of iodine-containing compounds of the peripheral tissues found; moreover, because the con-

centration of iodinated compounds in the plasma of DNP-treated animals is much lower than that of controls, the blood retained in the peripheral tissues at necropsy may also give rise to artifactual differences in the apparent tissue concentration of such compounds.

4. When the concentration of iodine-containing compounds in the tissues of perfused rats, killed at different intervals over a 12 hr period, was determined, it was observed that the over-all concentration of these compounds in peripheral muscle and brain remains the same in both DNP-treated rats and their controls, despite the much lower concentration in the plasma of the former.

5. Preliminary figures for the iodine content of total rat pituitaries showed it was the same in DNP-treated and control animals and that its variations parallelled those observed in brain and skeletal muscle.

6. The results obtained indicate that the plasma concentration of TCA-precipitable-iodine-containing compounds is not always a good index of alterations of the concentration of such compounds in the peripheral tissues.

7. The possible bearing of these findings are discussed in terms of the thyroid–pituitary feed-back system. The results reported in the present paper are tentatively interpreted as suggesting that the regulator(s) of thyroid–pituitary interrelationships are closely related to some intracellular parameter, alterations of which might be better reflected by changes in the concentration of iodine-containing compounds in the peripheral tissues, brain and pituitary included, than in the level of circulating total or 'free' thyroid hormone.

We are deeply indebted towards Professor A. Querido of the University of Leiden (Holland) for the encouragement and help given us, to Dr Rosalind Pitt-Rivers, F.R.S. (London), for criticism of the manuscript and to Professor L. van Middlesworth of the University of Memphis (U.S.A.) for the very useful suggestions with respect to isotopic equilibration of intact rats with radio-iodide.

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THE EFFECT OF 2,4-DINITROPHENOL ON THE 'UPTAKE' OF LABELLED THYROID HORMONES BY RED BLOOD CELLS AND RAT DIAPHRAGMS

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We have previously reported (Escobar del Rey & Morreale de Escobar, 1958) that at short intervals after the administration of radiothyroxine and 2,4-dinitrophenol to thyroidectomized, L-thyroxine-maintained rats, normal levels of radioactivity were found in the peripheral tissues, coexisting with a sharply decreased plasma PB^{131}I. (The following abbreviations are used: 2,4-dinitrophenol, DNP; ^{131}I-labelled L-thyroxine, ^{131}I-T_4, and L-triiodothyronine, ^{131}I-T_3; protein-bound iodine, PBI; thyroxine-binding globulin, TBG; thyroxine-binding albumin, TBA.) Moreover, an increased percentage of the blood radioactivity was in the red-blood-cell fraction in the DNP-treated rats. It has recently been shown (Morreale de Escobar & Escobar del Rey, 1960, 1961) that in intact DNP-treated rat the concentration of iodine-containing compounds in the tissues is maintained within limits comparable to those of untreated controls, whereas the plasma concentration is continuously low: the resulting increased tissue-to-plasma ratio of iodine-containing compounds is highest at short intervals of time after the administration of DNP, decreasing as the latter disappears from the circulation and increasing again after a new injection of the drug.

These findings suggested that the administration of DNP *in vivo* alters the distribution of thyroid hormones between plasma and tissues in favour of the latter. This paper reports results obtained when investigating whether such an effect is detectable by *in vitro* techniques and what mechanisms are likely to be involved. While this work was being carried out, reports appeared (Christensen, 1959, 1960*a*) describing a thyroxine-releasing effect of DNP on the thyroid-hormone–plasma-protein complex, with a dialysis technique. This paper shows that DNP does alter *in vitro* the distribution of radiothyroxine between plasma or plasma-free incubation

media and tissues, a direct effect of DNP on the cells being, however, postulated.

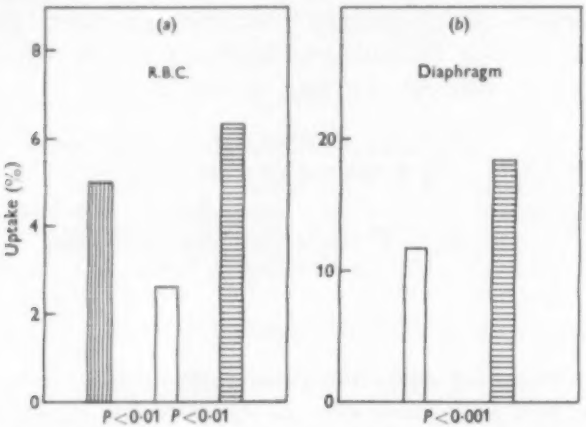

Fig. 1. Effect of DNP on the ^{131}I-T_4 'uptake' by rat R.B.C. and hemidiaphragms. (*a*) ^{131}I-T_4 was added in tracer amounts to whole blood of rats injected three times with 4 mg DNP at 8 hr intervals, to blood of untreated rats and to control blood to which 0·04 mg DNP/ml. was added. Incubation lasted 3 hr at 37° C. (*b*) ^{131}I-T_4 was added to a phosphate-saline buffer (Hogness *et al.* 1957) in tracer amounts; DNP in a 0·04 mg/ml. concentration. Incubation lasted 3 hr at 37° C. ▥ DNP-treated rats; □ control rats; ▤ control rats + DNP added *in vitro*.

METHODS

'*Uptake*' *of labelled thyroid hormones by red blood cells*: Determination of the 'uptake' was carried out by following in general a procedure similar to that proposed by Hamolsky, Stein & Freedberg (1957). The labelled hormones were added in tracer amounts, unless stated otherwise, to heparinized human or rat whole blood, the mixture was incubated at 37° C in stoppered tubes (Hamolsky, Golodetz & Freedberg, 1959) for 30 min–3 hr, the cells separated by centrifugation, washed thrice with ten volumes of isotonic saline and counted in a NaI (Tl) well-type scintillation counter. Both L-Thyroxine labelled with ^{131}I in the 3′ and 5′ and triiodo-L-thyronine in the 3′ positions, were supplied by Abbott Laboratories of Oak Ridge (U.S.A.), purified, whenever necessary, immediately before use on an anion-exchange resin and checked by chromatography.

RESULTS

Under such experimental conditions it was repeatedly observed that DNP, whether added *in vitro* or injected *in vivo*, increased the 'uptake' of labelled thyroxine both by human and rat R.B.C., typical results being shown in Fig. 1. It also increases the 'uptake' of labelled triiodo-L-thyronine, though proportionally less than that of ^{131}I-T_4: using whole rat blood incubated for 30 min with tracer amounts of ^{131}I-T_4 or ^{131}I-T_3, the

mean 'uptakes' were 3·4% in the absence and 9·3% in the presence of 0·1 mg DNP/ml. ($P < 0·001$) when L-T_4 was used, 20·0 and 34·5% respectively ($P < 0·001$) for L-T_3.

Such an effect of DNP could well be mediated by interference by the drug on the thyroid-hormone–plasma-protein complex, by a direct action on cells, or by both. In order to investigate this point an attempt was first made to study the effect of DNP on the ^{131}I-T_4 'uptake' by washed R.B.C. incubated in protein-free media. The procedure, however, was found unreliable. In fact, it seems that R.B.C. integrity is not maintained; despite use of quite different isotonic buffers and media there was always some degree of haemolysis at the end of incubation and the control 'uptake' figures were in the order of magnitude described for R.B.C. stroma (Crispell & Coleman, 1956). Moreover, in the absence of binding sites in the media, adsorption of labelled thyroxine to glass-ware and specially to the large surface presented by the R.B.C. introduced more uncontrollable variables.

Owing to these difficulties indirect evidence was sought. Since it was found that repeated washings and incubation in media containing low concentrations of plasma proteins eliminated such difficulties, the problem was investigated by using R.B.C., separated from plasma, washed once with ten volumes of saline and twice with ten volumes of saline containing 1% rat plasma and then incubated with the labelled hormone and DNP for 30–60 min in saline containing from 1 to 5% rat plasma. After incubation the R.B.C. were separated and washed as usual. Increased ^{131}I-T_4 'uptakes' by the DNP-treated R.B.C. were always found. This, however, did not clarify the point, and an attempt was made to circumvent the necessary presence of protein in the system by (*a*) using very high thyroxine concentrations, which should be more than enough to saturate specific binding sites on the plasma proteins, and thus to minimize the effect of any further increase of the already high 'free' thyroxine fraction; (*b*) maintaining the plasma protein concentration constant and altering the concentration of R.B.C. If DNP acted by increasing the 'free' thyroxine level in the incubation medium, the line resulting when the 'uptake' is plotted against the R.B.C. content should be parallel and with a higher intercept than that obtained in the absence of the drug.

Figure 2(*b*) shows results obtained when R.B.C. treated as indicated above were incubated 60 min in saline containing 2·5% rat plasma and increasing thyroxine concentrations. DNP (0·10 mg/ml.) increased the ^{131}I-T_4 'uptake' even at the highest hormone concentration employed. Curves obtained with an incubation medium containing only 1·0% rat plasma were parallel.

Three series were prepared with saline containing 1·25, 2·5 and 5·0% rat plasma as incubation medium and ^{131}I-T_4 in tracer amounts; the

'uptake' was determined for three different R.B.C. concentrations for each series, namely 12, 24 and 45% of the total volume. When the 'uptake' data were plotted against the corresponding R.B.C. content for each plasma-protein concentration, three parallel lines were obtained. The line with the highest intercept corresponded to the series with the lowest plasma-protein content, and, presumably, the highest proportion of 'free' thyroxine. When 0·10 mg DNP/ml. was added, the line obtained had a higher intercept than that corresponding to the same plasma-protein concentration, but the slope was steeper.

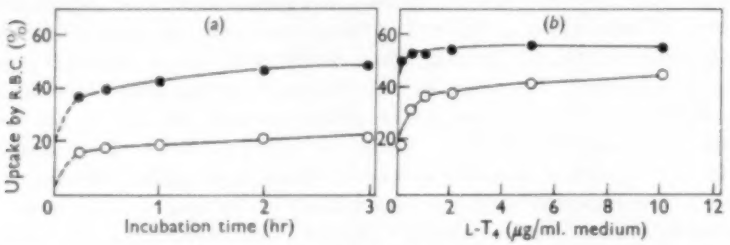

Fig. 2. Effect of DNP on the 'uptake' of ^{131}I-T_4 by washed R.B.C. incubated in saline containing rat plasma. (*a*) Influence of 0·10 mg DNP/ml. on the 'uptake' by R.B.C. incubated for 20 min–3 hr in saline containing 5% rat plasma and tracer doses of labelled thyroxine. (*b*) Influence of 0·10 mg DNP/ml. on the 'uptake' by R.B.C. incubated 60 min in saline containing 1% rat plasma and increasing concentrations of L-thyroxine. ○ control; ● DNP added.

The increased 'uptakes' obtained with DNP at the time intervals studied might have been due to the fact that the drug merely accelerated attainment of a maximum which would eventually be reached in the absence of the drug. Figure 2(*a*) shows results obtained with the above procedure, using saline containing 5% rat plasma as incubation medium, tracer amounts of ^{131}I-T_4 and increasing incubation periods. As may be seen, DNP actually maintains the 'uptake' at a higher level. It was concluded from this type of procedure that the data could not be explained by an action of DNP on the thyroid-hormone–plasma-protein complex only, a direct action on the R.B.C. being more likely.

Further experiments were carried out as follows. Whole rat blood was incubated for 30 min at 37° C with or without DNP (usually 0·1 mg/ml.), the R.B.C. were then separated by centrifugation, washed once with ten volumes of saline and twice with ten volumes of saline containing 1% rat plasma. They were incubated for another half-hour at the same temperature in saline containing 2·5% rat plasma and ^{131}I-T_4 in tracer amounts, after which the cells were separated and washed as usual. It was found that the 'uptake' of ^{131}I-T_4 by R.B.C. which had been pre-incubated with

DNP was always appreciably higher than that corresponding to untreated cells: i.e. 26% for controls versus 40% for DNP-pre-incubated R.B.C. ($P < 0.001$). Figure 3 shows that an increased 'uptake' was already appreciable at very low concentrations of DNP (0·0025 mg/ml.) during pre-incubation. These results also suggest a direct action of the drug on the R.B.C.: it seems rather unlikely that the traces of DNP, if any, which might be left in the incubation medium containing the labelled hormone after repeated washings might be responsible for the observed effect by acting on the small amounts of plasma proteins present.

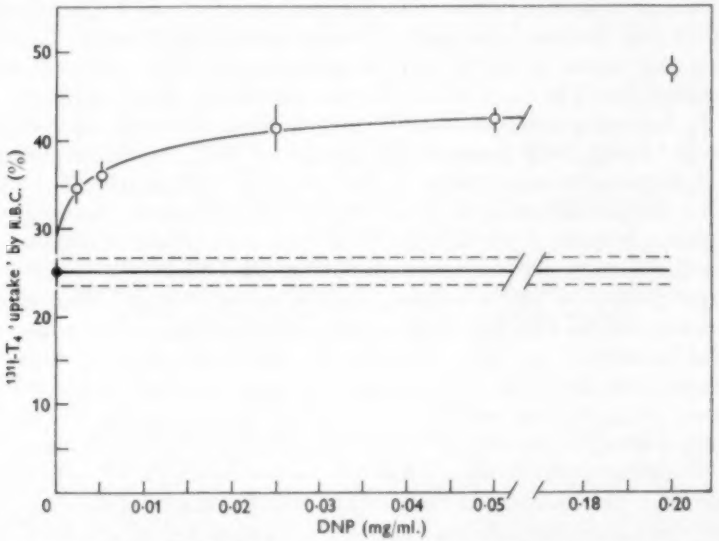

Fig. 3. Influence of DNP, 0·0025; 0·005; 0·025; 0·050 and 0·200 mg/ml., on the ^{131}I-T_4 'uptake' by R.B.C. pre-incubated 30 min with the drug and repeatedly washed before incubation for 30 min in contact with the labelled hormone in saline containing 2·5% rat plasma. Means ± S.D. of three samples are given. ● no DNP; ○ DNP.

'Uptake' of labelled L-*thyroxine by rat hemidiaphragms*

In general the procedure described by Hogness, Lee, Berg & Williams (1957) was followed. Young adult male or female Wistar rats were exsanguinated under ether narcosis through the inferior vena cava after injection of heparin. The diaphragms were rapidly excised, cleaned of adhering tissue, divided into halves, rinsed in incubation medium, blotted on filter paper and weighed on a torsion balance to the nearest milligram. They were transferred quickly to the incubation vessels, one hemidiaphragm serving as control, the other for DNP treatment, right and left being alternated. The incubation medium was either saline, saline-phosphate buffer (Hogness *et al.* 1957), Krebs-Ringer-phosphate or K-R-P+glucose (1 mg/ml.), pH 7·2–7·4, to which ^{131}I-T_4 was added in tracer amounts unless stated otherwise. DNP was usually added at a concentration of 0·05–0·1 mg/ml. Incubation was carried out

for 30 min–3 hr at 37° C in stoppered vessels, after which the tissue was rinsed thrice for 30 sec in ten volumes of cold incubation medium, blotted on filter paper and counted. All data are given as percentage of added ^{131}I-T_4 remaining with the hemidiaphragm per 100 mg wet weight. For some experiments the animals were perfused at killing with 100–150 ml. isotonic saline.

Figure 1 shows typical results. DNP increases the 'uptake' of ^{131}I-T_4 by rat hemidiaphragms in the absence of added plasma proteins. Similar results were obtained when using hemidiaphragms from saline-perfused rats: 11·4 % for controls versus 17·0 % for DNP-treated tissues ($P < 0{\cdot}001$). It was observed that the response of the 'uptake' to DNP was of the same intensity irrespective of the incubation medium used. The response to DNP was, however, abolished by boiling the hemidiaphragms. Figure 4 shows the results obtained with hemidiaphragms from perfused rats, incubated for 3 hr in K-R-P + glucose containing tracer amounts of ^{131}I-T_4, increasing concentrations of stable hormone and 0·05 mg DNP/ml. As may be seen, DNP increases the 'uptake' of ^{131}I-T_4 by the diaphragms at all hormone concentrations studied, even for 1000 μg/100 ml. buffer. Such a concentration should be sufficient to saturate specific binding sites on plasma proteins if the incubation medium were totally substituted by plasma. Since the experimental conditions used make the presence of plasma proteins in the incubation medium rather unlikely, these results again support the idea that DNP exerts a direct action on the tissue.

The technique was later modified by pre-incubating the hemidiaphragms with DNP for 2·5 hr, followed by four washings with a tenfold volume of incubation medium and a second incubation for 2 hr in the presence of tracer amounts of ^{131}I-T_4. The 'uptakes' of labelled hormone by the hemidiaphragms which had been pre-incubated with DNP were higher than those corresponding to the controls, whether the incubation medium used was saline + 1 % plasma (15·9 % for controls and 21·5 % for DNP-pre-incubated, $P < 0{\cdot}01$) or K-R-P + glucose (18·3 and 23·7 % respectively, $P = 0{\cdot}02$). An increased ^{131}I-T_4 'uptake' by DNP-pre-incubated hemidiaphragms was also obtained with a protein-free K-R-P + glucose buffer and tissue from thoroughly perfused rats. These results are difficult to interpret on the basis of an action of DNP on the binding of thyroxine to plasma proteins.

The response to DNP observed in these pre-incubation experiments was less intense than that obtained with diaphragms directly incubated with DNP and ^{131}I-T_4 in the first incubation medium; this was to be expected, considering the long pre-incubation period and more frequent handling of the tissue.

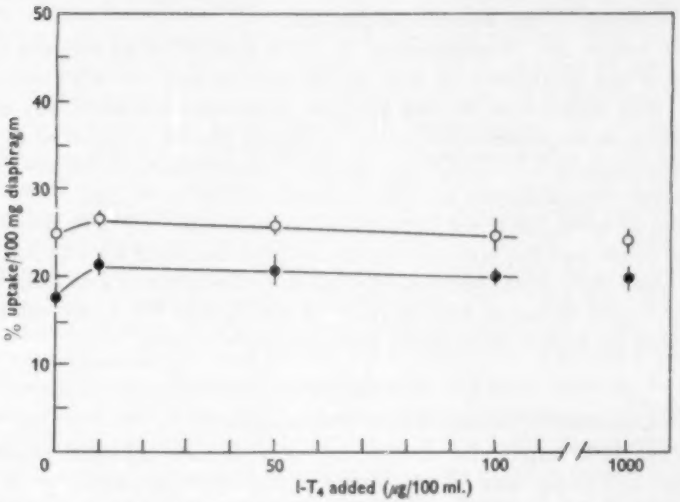

Fig. 4. The effect of DNP 0·05 mg/ml. on the ^{131}I-T_4 'uptake' by hemidiaphragms from thoroughly perfused rats, incubated for 3 hr in Krebs–Ringer-phosphate + glucose (1 mg/ml.) buffer, pH 7·2–7·4, containing increasing concentrations of L-thyroxine. The mean ± S.D. is given. ● no DNP; ○ DNP.

Electrophoretic studies

For this purpose human serum was mostly used, though some determinations were also carried out with rat sera. Untreated sera, and sera to which DNP was added in concentrations (0·05–0·10 mg/ml.) which induce an increase in the ^{131}I-T_4 'uptake' by R.B.C. to double or more of the control value, were separated by electrophoresis on Whatman No. 1 or No. 3 paper in a closed system apparatus. Either sodium 5,5-diethylbarbiturate-5,5-diethylbarbituric acid buffer, ionic strength 0·05, pH 8·6, or sodium 5,5-diethylbarbiturate-sodium acetate-sodium chloride buffer, molal ratio 2:1:1, adjusted to pH 8·6 with 1 N-HCl, ionic strength 0·1, was used. The latter buffer (Levey & Roberts, 1958) gave a very good separation of the α_1-globulin from albumin and was always used for rat serum. ^{131}I-T_4 was added in tracer amounts or with increasing concentrations of hormone in order to determine by a relative distribution method (Dowling, Freinkel & Ingbar, 1956) whether the presence of DNP altered the binding capacity of the plasma proteins. In one case DNP was added to the electrophoretic buffer and the distribution of the radioactivity between TBG and TBA was compared with that of the same plasma run simultaneously in buffer not containing the drug. Ingbar (1960) has recently

shown an 'unbinding' effect of salicylate by this procedure, which had not been detected by the standard techniques.

The yellow spot corresponding to DNP was found to migrate much ahead of the albumins both with human and rat sera. We were unable to show with either type of sera and the procedures described above any difference in the distribution of ^{131}I-T_4 among plasma proteins or in their binding capacity when DNP was present. To ascertain that the procedures employed were adequate to disclose abnormalities in binding of ^{131}I-T_4 to plasma proteins, butyl-4-hydroxy-3,5-diiodobenzoate (BHDB) was added to rat serum and the same alterations as those described by Van Arsdel & Williams (1956) were observed. BHDB at concentrations similar to those of DNP also increases the 'uptake' of ^{131}I-T_4 and ^{131}I-T_3 by rat R.B.C. (Escobar del Rey & Morreale de Escobar, unpublished).

DISCUSSION

From the results described here it is concluded:

(1) That DNP alters the distribution of thyroxine between plasma or protein-free media and cells in favour of the latter, as shown by *in vitro* techniques.

(2) It is suggested that this effect of DNP is responsible for the distribution of iodine-containing compounds found *in vivo* in rats (Morreale de Escobar & Escobar del Rey, 1961).

(3) It seems unlikely that such an effect is mainly and/or solely mediated by an alteration of the binding of the hormones to plasma proteins and it is postulated that some as yet undetermined cellular mechanism is directly involved.

The latter point is strongly supported by the data presented here. On the one hand, an effect of DNP on the thyroid-hormone–plasma-protein complex has not been demonstrable by means of electrophoretic techniques which were sufficiently sensitive to disclose another known alteration (Van Arsdel & Williams, 1956). On the other, an intense effect of the drug on the 'uptake' of thyroid hormones by rat R.B.C. and diaphragms has repeatedly been shown under experimental conditions which exclude a mechanism operating only by an action on plasma proteins.

However, Christensen (1959, 1960*a*), using a dialysis procedure, has found that the rate of transfer of ^{131}I-T_4 across the membrane is increased when DNP is added to the plasma, and interpreted this finding as an indication of a thyroxine-releasing effect of the drug on the thyroxine–plasma-protein complex. Closer inspection of his data indicates that the increase in rate of transfer of radioactivity across the membrane is rather weak as compared with the intense effect of equal doses of DNP on ^{131}I-T_4 and ^{131}I-T_3 'uptake' by R.B.C. reported here. Christensen (1960*b*) has in

fact reported a good correlation between the intensity of the changes in 'free' thyroxine and in ^{131}I-T_3 R.B.C. 'uptake', using serum from patients with various thyroidal conditions and pregnant women. With salicylate (Christensen, 1960*c*) the dose resulting in a 100% increase above normal of the ^{131}I-T_3 'uptake' by R.B.C. augments the rate of transfer of labelled I-T_4 across the dialysis membrane 200% above normal. On the contrary, in the case of DNP, doses (0·005 mg/ml.) which have been found by us to increase the R.B.C. 'uptake' of ^{131}I-T_4 and ^{131}I-T_3 about 100% or more affect the rate of transfer only by 9%; a 38% increase in the rate of transfer of radiothyroxine was elicited by 0·05 mg DNP/ml., a dose which results in a 300% increase in the ^{131}I-T_4 'uptake' by R.B.C. It has been reported in a recent publication (Christensen, 1961) that the addition to the dialysis system of tris-hydroxymethylaminomethane (Tris), barbital and urea, the latter in doses too low for denaturation of proteins, resulted in increased rates of transfer of ^{131}I-T_4 across the membrane, up to 322% above normal with urea. As Christensen (1961) points out, it is difficult to interpret some of the results obtained with this latter compound as the result of a 'thyroxine-releasing' effect, and other possible causes cannot be excluded, i.e. that the membrane is affected. Rates of transfer of ^{131}I-T_4 across the dialysis membrane in the procedure used by Christensen are strictly comparable only when all experimental conditions are held constant, the serum being the only variable.

Therefore, there is direct evidence that DNP exerts an intense influence on the 'shift' of thyroid hormones towards tissues even in the absence of plasma proteins; an 'unbinding' effect on the thyroid-hormone–plasma-protein complex, on the contrary, appears as rather weak, if any, by equilibrium dialysis (Christensen, 1959) and is not demonstrable by electrophoretic procedures. We believe the facts discussed above lend experimental support to the last of our conclusions. No comments may be reliably formulated from the present data about the nature of the mechanisms involved at a cellular level which result in an increased 'uptake' of thyroxine.

At present one cannot be sure that a mechanism detected *in vitro* is the one operating *in vivo*. However, in the present instance the same effect was observed with similar intensity whether DNP and the labelled hormone were both administered *in vivo* (Escobar del Rey & Morreale de Escobar, 1958), the drug *in vivo* and the hormone *in vitro*, or both *in vitro*. Moreover, the tissue-to-plasma ratio of iodine-containing compounds increases for DNP-treated rats (Morreale de Escobar & Escobar del Rey, 1961) with an intensity of the same order of magnitude as the *in vitro* effect of DNP. It was thus concluded (Morreale de Escobar & Escobar del Rey, 1961) that it is very likely that the distribution pattern of thyroid hormone found for

intact animals is brought about by a 'shift' of the hormone towards the tissues caused by a direct effect of DNP on cells.

The bearing of such conclusions on the mechanism leading to intracellular concentration and metabolism of iodine-containing compounds, and the possible role of such parameters in a thyroid-pituitary feed-back system are discussed elsewhere (Morreale de Escobar & Escobar del Rey, 1961).

SUMMARY

1. Both injection of DNP and addition of the drug to whole blood *in vitro* increase the 'uptake' of ^{131}I-T_4 and ^{131}I-T_3 by rat and human R.B.C. The 'uptake' of ^{131}I-T_4 by washed R.B.C. incubated with the labelled hormone in saline containing 1–2·5% rat plasma and increasing concentrations of stable thyroxine (up to 1000 μg/100 ml.) is also higher when DNP is present in the medium. When R.B.C. are pre-treated with DNP and washed repeatedly before incubation with ^{131}I-T_4, the resulting 'uptake' is higher than the uptake by controls even with very low initial concentrations of DNP, 0·0025 mg/ml.

2. Addition of DNP to protein-free incubation media results in an increased 'uptake' of ^{131}I-T_4 by rat hemidiaphragms, even when the tissues were obtained from perfused rats and incubated with high concentrations of L-thyroxine (up to 1000 μg/100 ml. buffer). Pre-incubation of the hemidiaphragms with DNP, followed by repeated washings of the tissue before incubation with ^{131}I-T_4 also increases the 'uptake'.

3. The use of several electrophoretic procedures, both with human and rat sera, did not disclose any effect of DNP on the distribution of ^{131}I-T_4 among plasma proteins or in their binding capacity.

4. It is concluded that DNP alters the distribution of thyroid hormones between plasma or incubation media and tissues in favour of the latter. It is postulated that a direct effect of DNP on the cells is involved and not mainly and/or solely an alteration of the thyroid-hormone–plasma-protein complex. It is considered likely that this 'shift' of the thyroid hormone towards tissues is responsible for the distribution pattern of iodine-containing compounds found *in vivo*.

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THE EFFECT OF PROGESTERONE AND OXYTOCIN ON THE MOUSE MYOMETRIUM

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Studies of the excitability of the uterus are complicated by the regulatory effects of the ovarian hormones which determine its functional state. For example, the motility of the pregnant rabbit uterus can be completely inhibited by endocrine control, a phenomenon called 'inactivation' by Reynolds (1949) and 'progesterone block' by Csapo (1956*a*, *b*).

The effects of progesterone on the contractile mechanism of the rabbit's myometrium have been analysed quantitatively *in vitro* by Csapo & Corner (1952, 1953), and *in vivo* by Schofield (1954, 1955, 1957, 1960) using isometric techniques. Treatment with progesterone *in vivo*, with or without subsequent administration of oestrogen, induces hyperpolarization of the muscle fibre membrane in the pregnant and non-pregnant rabbit (Goto & Csapo, 1957, 1959) and in the castrated rat (Marshall, 1959). Treatment with progesterone *in vitro* also blocks the discharge of action potentials and increases the membrane potential in the pregnant and postpartum rat myometrium (Kuriyama & Csapo, 1961*b*; Marshall & Csapo, 1961).

The membrane potential in the pregnant myometrium is higher at placental than at non-placental sites; this has been shown in the rabbit (Goto & Csapo, 1959), the rat (Thiersch, Landa & West, 1959; Kuriyama & Csapo, 1959*b*) and in the mouse (Goto, Kuriyama & Abe, 1959, 1960). A block of propagation at placental regions, while the non-placental portion was still propagating, was observed in the pregnant rabbit myometrium (Kuriyama & Csapo, 1961*a*).

During the first part of gestation the rabbit myometrium shows, as a rule, an absence of spontaneous discharge and of response to oxytocin. In the later stages of pregnancy (30th–31st days), action potentials are discharged infrequently and the sensitivity to oxytocin increases. The mouse and rat myometrium generate spontaneous discharges and respond to oxytocin some days earlier than does the rabbit uterus.

The membrane characteristics of the pregnant mouse myometrium driven electrically have been observed by Goto, Kuriyama & Abe (1959, 1961).

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Further investigations on the mouse myometrium have now been made, including the effects of progesterone and of oxytocin. The mechanism of action of progesterone in the maintenance of pregnancy is also discussed.

METHODS

Myometrium from mice in the 13th–15th and 18th–20th days of pregnancy, and 3–6 hr post-partum was used; the gestation period in mice is 20–21 days. The uterus was excised under ether anaesthesia and placed in oxygenated mammalian Krebs's solution at room temperature. The composition of the Krebs's solution was (mM): NaCl 118·5, $NaHCO_3$ 24·9, KCl 4·7, $CaCl_2$ 2·5, KH_2PO_4 1·2, $MgSO_4$ 1·2; +glucose 1 g/l. It was oxygenated with 95 % O_2 and 5 % CO_2.

The uterus was dissected into strips 3 mm wide and 30 mm long (*in vivo* lengths) for measurements of membrane activity, and 5 mm wide and 30 mm long for measurement of isometric contractions, which were recorded by the method used by Coutinho & Csapo (1959) on rabbit myometrium.

Membrane electrical activity was recorded by the method of Kuriyama & Csapo (1961*b*). The micro-electrodes were glass capillaries with a resistance of 20–30 MΩ, mounted flexibly by Woodbury & Brady's (1956) method. The insertion of the electrode into a single cell was controlled by a micro-manipulator. The tension was recorded by a strain gauge, simultaneously with the action potential. The membrane and action potentials and the deflexion of the gauge were amplified and recorded by a cathode-ray oscilloscope (Tektronix model 502).

Solutions of progesterone up to 10 μg/ml. were made in Krebs's solution; for higher concentrations the drug was dissolved in propylene glycol and diluted with Krebs's solution. Oxytocin was dissolved in Krebs's solution.

The experiment was started a few minutes after removal of the uterus; the rest of the uterus was kept in cold oxygenated Krebs's solution for later experiments.

RESULTS

Effect of progesterone on tension

A strip from the post-partum uterus of the mouse (3–6 hr after delivery) develops rhythmic changes in tension (range 9–15 g, mean 12 g); these contractions continue for long periods without electrical or chemical stimulation. In the myometrium of the pregnant mouse there is more variation in the magnitude of tension developed (range 5–18 g, mean 11 g), and the duration of the contraction-relaxation cycles (mean 23 sec) is shorter than in the post-partum myometrium (mean 31 sec).

In the post-partum mouse myometrium, as in that of the rabbit, rat and man (Csapo 1956, 1961), *in vitro* treatment with progesterone 5, 10 or 20 μg/ml. abolished contractions. The decrease in tension and the duration of the effect were proportional to the concentration of progesterone. The frequency of contractions was initially and transiently increased. After washing out the progesterone with Krebs's solution, recovery of tension was quick and complete. The effects are illustrated in Fig. 1.

In the late-pregnant mouse myometrium (17th–20th days), treatment with progesterone also increased the frequency of contractions initially,

but reduced the magnitude of tension developed and finally led to irregular contractions. The time taken for the abolition of contractions was longer in the pregnant (mean 28 min in 20 μg/ml.) than in the post-partum state (mean 8 min in 20 μg/ml.). The time of recovery after washing out the

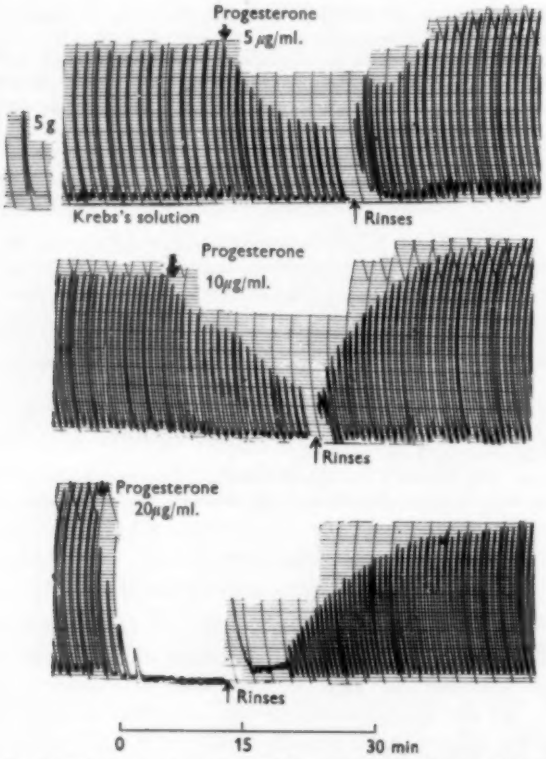

Fig. 1. Longitudinal muscle of mouse myometrium 6 hr post-partum. Upper, middle and lower traces show effect on motility of progesterone 5, 10 and 20 μg/ml., respectively, applied at arrows. 'Rinses' indicates washing three times with Krebs's solution. Tension scale in grams in this and all subsequent tracings.

progesterone was also longer (mean 55 min in the pregnant, and 25 min in the post-partum, myometrium).

Calcium-deficient Krebs's solution (less than 0·25 mM or calcium-free Krebs's solution) had an action very similar to that of progesterone on the isometric tension of the mouse myometrium. Its effect developed also more rapidly in post-partum than in pregnant myometrium, as observed by Coutinho & Csapo (1959) for the rabbit uterus.

Effect of oxytocin on tension

The sensitivity to oxytocin of the tension developments of the myometrium is entirely dependent upon the hormonal domination. A progesterone-dominated uterus (13th–15th days of pregnancy) shows contractions of irregular frequency and magnitude. The mean value of the frequency was 0·4 c/min, whilst the mean contraction tension was 5·5 g; the resting tension (tonic contraction) remained zero. Oxytocin 5 μu./ml. produced no effect and 50 μu./ml. slightly increased the active tension to 7 g; oxytocin 500 μu./ml. increased the frequency of active tension to 0·9 c/min and its magnitude to 9·5 g, and the resting tension to 1·1 g. At the 18th day of pregnancy (termination of progesterone domination) the frequency of spontaneous active tension development became 0·8 c/min and the magnitude of active tension 9·5 g; oxytocin 5 μu./ml. only increased the magnitude to 12 g. Oxytocin 50 μu./ml. increased the frequency to 1·1 c/min, the magnitude to 11 g and the resting tension to 0·5 g. Oxytocin 500 μu./ml. increased the frequency of the active tension to 1·9 c/min, the magnitude to 10·5 g and the resting tension to 1·3 g. Six hours post-partum (under oestrogen domination) the frequency of the spontaneous active tension development was 1·2 c/min and the magnitude 10 g. Oxytocin 5 μu./ml. increased the frequency to 1·6 c/min, the magnitude to 13 g; 50 μu./ml. increased the frequency to 2·5 c/min, the magnitude to 11 g and the resting tension to 2 g. With oxytocin 500 μu./ml. the frequency increased to 2·7 c/min, the magnitude decreased to 8 g and the resting tension increased to 6 g. The maximum sensitivity to oxytocin of the mouse myometrium appears either during delivery or shortly afterwards, having increased progressively during gestation. In the pregnant myometrium the irregularity of contractions continued in the presence of oxytocin and the increase in the resting tension was less than in the post-partum myometrium. Sometimes the post-partum myometrium showed irregular periods of contractions, separated by long silent periods. These irregular periodic contractions were not greatly affected by oxytocin, though there was some increase in the magnitude and in the number of contractions which occurred in each train.

When progesterone was given in the above condition of the myometrium, a regular pattern appeared before the contractions were abolished. After rinsing with Krebs's solution to remove progesterone as well as oxytocin the activity always returned to a regular, but never again to the irregular, period pattern.

Comparisons were made of the effect of progesterone alone with that of simultaneous treatment with progesterone and oxytocin on post-partum mouse myometrium. Progesterone 10 μg/ml. in the presence of oxytocin

50 μu./ml. blocked the activity. This effect was, however, preceded by a period during which the frequency of contractions was increased more than by either substance acting alone, and the resting tension was also increased more than with oxytocin alone, even though progesterone by itself lowered the resting tension. Higher concentrations of progesterone and oxytocin given together rapidly blocked activity and increased the resting tension.

Spontaneous membrane activity in the myometrium

The membrane and action potentials in the myometrium vary considerably under different endocrine conditions. Table 1 shows the magnitude of membrane and action potentials, the number of spikes in a train of discharges, and the intervals between spikes and trains of discharges. The size and shape of the action potentials in pregnant mouse myometrium driven electrically have been described by Goto *et al.* (1959). In general the variations in membrane potential values are smaller in the post-partum than in the pregnant myometrium.

TABLE 1. Membrane (*MP*) and action (*AP*) potentials at 35° C in the pregnant and post-partum mouse myometrium

Condition of myometrium	No. of specimens	*MP* (mV)				*AP* (mV)				50% duration (msec)			
		Mean	Range	S.D.	S.E.	Mean	Range	S.D.	S.E.	Mean	Range	S.D.	S.E.
18–20th day pregnancy	18	53	42–69	5·8	±0·8	42	29–55	5·9	±0·8	31	18–45	4·8	±0·6
Post-partum 6 hr	11	46	38–62	4·6	±0·7	41	31–58	4·4	±0·7	38	21–58	5·2	±0·8

	Interval between first spike and second in a train (msec)		No. of spikes in one train discharge		Interval between two train discharges (min)	
	Mean	Range	Mean	Range	Mean	Range
18–20th day pregnancy	500	250–800	28	9–48	29	6–47
Post-partum 6 hr	750	400–1200	21	11–35	37	8–62

Figure 2 shows recordings of spontaneous activity in a post-partum mouse myometrium. In contrast to rat myometrium (Kuriyama & Csapo, 1961*b*), the membrane of the mouse myometrium remains continuously depolarized during a train of discharge, the first spikes of a train being generated from the steady level of membrane potential, but successive spikes arising before complete repolarization has occurred. The relative refractory periods of the myometrium were measured by Goto *et al.* (1959) and found to be shorter in mice than in guinea-pigs, rats and cats (Bozler, 1938, 1940). Also the frequency of spike discharge is higher in the mouse than in the rat (Kuriyama & Csapo, 1961*b*). The continuous depolarization of the membrane may therefore be due to the shorter refractory period of the mouse myometrium.

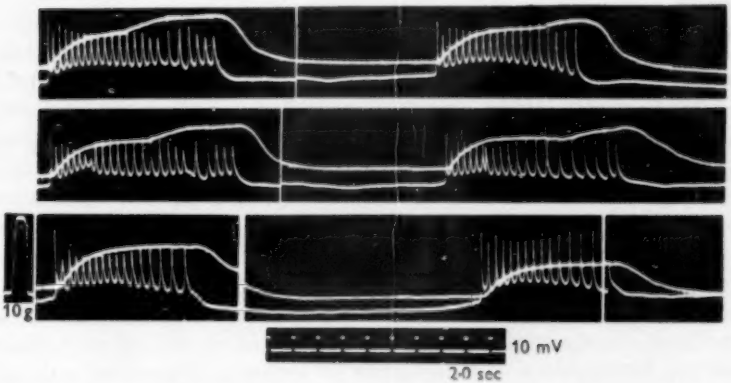

Fig. 2. Post-partum myometrium of mouse (6 hr). Simultaneous recording of action potentials from a single fibre and of the myometrial tension. Upper, middle and lower traces are recorded from the same cell. Each contraction differs in shape.

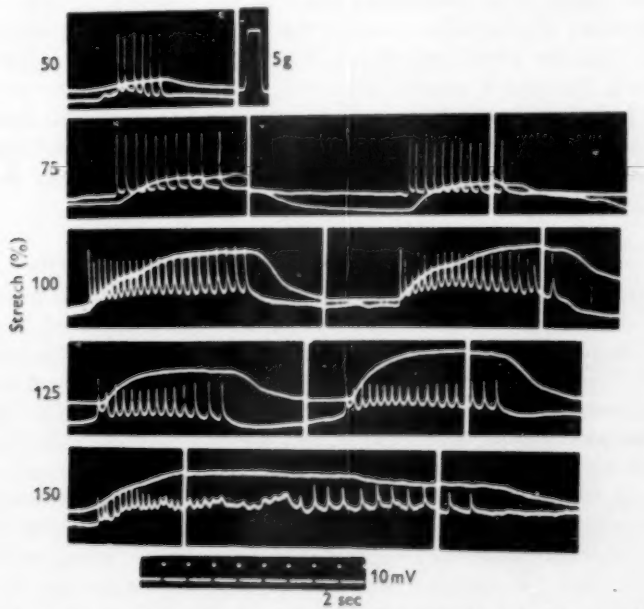

Fig. 3. Effect of stretch on membrane activity and tension (continuous line) in post-partum mouse myometrium (6 hr); 50, 75, 100, 125 and 150% stretch (see text).

Effect of stretch

Figure 3 shows the effect of stretching the myometrium. The length of a strip of post-partum mouse myometrium was fixed at 50, 75, 100, 125, and 150% stretch, 100% stretch (the resting length) being defined as the length at which slight change in resting tension occurs but changes in active tension are maximum (Csapo & Goodall, 1954; cf. Hill, 1939). At each degree of stretch changes in membrane activity and tension were recorded simultaneously. At 50% stretch there were a few spikes in each train discharge, and their frequency was low. Increasing the stretch caused an increase in membrane activity, with depolarization and an increase in the number of spikes in a train; at 150% stretch there was significant depolarization of the membrane, continuous spike discharge, and the development of tension was no longer synchronized with spike generation in the single cell from which records were taken. The increase in the resting tension after stretching became less after 3–15 min, but it remained higher than at 100% length. The magnitude of the increase in resting tension and the time taken for it to fall from its peak value to a constant raised value were proportional to the degree of stretch. In the overstretched myometrium (150%) the increase in resting tension was greater than the active tension, i.e. the resting tension was significantly increased, but active tension decreased. Values of membrane potential, action potential, resting tension and active tension at various degrees of stretch are illustrated in Fig. 4. The effect of stretch in mouse myometrium was almost the same as in the rat, as found by Kuriyama & Csapo (unpublished).

Effect of progesterone and oxytocin on membrane activity

Progesterone 10 μg/ml. in Krebs's solution decreased the number of spikes discharged and the magnitude of the spikes. After 10–20 min of progesterone treatment the propagation of the spikes was blocked, and only electrotonic or local potentials remained. As the number of spikes in a train decreased, the development of tension also decreased. This blocking of spike propagation developed more slowly in pregnant than in post-partum myometria. Figure 5 illustrates the effect of progesterone in pregnant mouse myometrium, similar to that observed in rat and rabbit myometrium (Kuriyama & Csapo, 1959*b*, 1961*b*). The changes in membrane potential caused by progesterone appeared very slowly, and always occurred after the abolition of excitability of the membrane. A hyperpolarization of 10 mV was seen 30–40 min after the application of progesterone in post-partum myometrium, and 3–5 mV hyperpolarization after 50–60 min in pregnant myometrium. Thus progesterone first increased

the threshold of excitability and blocked spike propagation; it then abolished spontaneous activity, and finally the membrane became hyperpolarized.

The effects of oxytocin on the membrane in the pregnant and postpartum mouse myometrium were found to be the same as those in rat myometrium (Kuriyama & Csapo, 1959*b*, 1961*b*; Goto, 1960). Oxytocin in low concentration (50 μu./ml.) increased the frequency of spike discharges in the middle stage of gestation, but did not regularize the size of

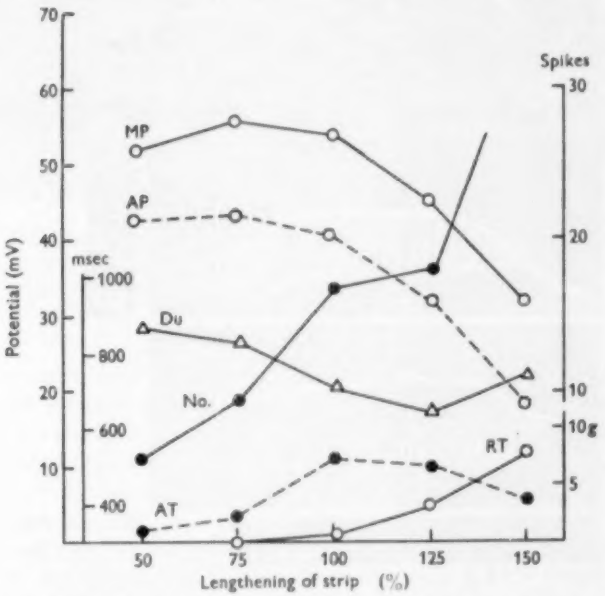

Fig. 4. Effect of stretch on membrane activity and tension development in postpartum mouse myometrium. Membrane activity recorded from single cell and tension changes from the whole tissue. MP, membrane potential (mV), ○——○; AP, action potential (mV), ○- - -○; No., number of discharges in a train, ●——●; Du, duration between first and second spike discharge in a train (msec), △——△; AT, active tension (g.), ●- - -●; RT, resting tension (g), ○——○.

action potentials. In the last stage of gestation the membrane potential was lowered by treatment with oxytocin 50 μu./ml. and the frequency and the number of spike discharges were increased; also the size of the spikes was somewhat regularized. Synchronization of contraction with spike generation during these stages of gestation was accelerated by treatment with oxytocin. Oxytocin in higher concentrations (500 μu./ml.) decreased the membrane potential and increased the frequency and the number of

spike discharges, but often blocked spike generation in a highly depolarized membrane and left the local potentials in the last stage of train discharges. Higher concentrations of oxytocin induced periodic oscillatory action potentials. Six hours post-partum, frequency was increased by treatment with oxytocin 1 μu./ml. and the effects of 5 μu./ml. were nearly comparable

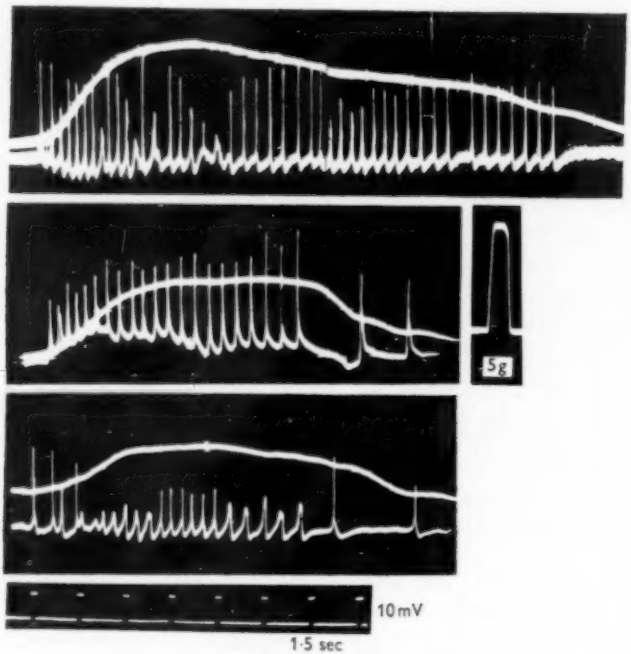

Fig. 5. Pregnant mouse myometrium (20th day). Effect of progesterone on membrane activity, and on tension (continuous lines). Upper trace: control spontaneous spike discharges and contraction. Middle trace: 10 min after progesterone 10 μg/ml. Lower trace: after 20 min. The membrane potential is raised by treatment with progesterone, which blocks the propagation of the spike discharges but leaves the local potential.

to those produced by 500 μu./ml. in the middle stage of gestation and by 100 μu./ml. in the last stage of gestation; oxytocin 100 μu./ml. induced long-continued spike generation in the depolarized membrane, leading to the oscillatory action potentials. The size of the action potentials decreased and their duration was prolonged. The frequency of spike discharges decreased compared with that seen after treatment with oxytocin 50 μu./ml. Figure 6 shows the effect of oxytocin (50 μu./ml.) on a mouse myometrium

in the latest stage of gestation. The membrane is depolarized (6 mV) and the frequency, number of spike discharges and magnitude of contraction is increased.

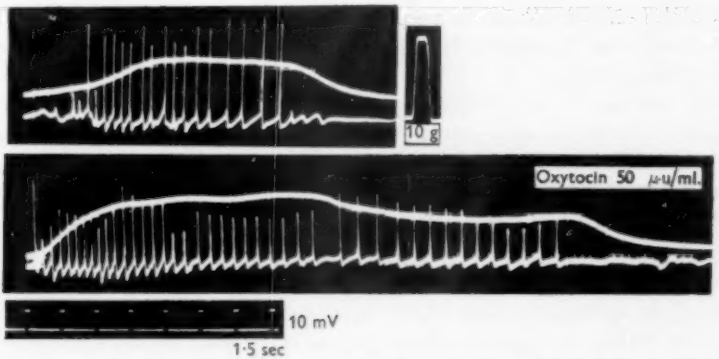

Fig. 6. Pregnant mouse myometrium (20th day). Effect of oxytocin on membrane activity and on tension (continuous line). Upper trace: control spontaneous discharges and tension development. Lower trace: after 3 min treatment with oxytocin.

DISCUSSION

In the mouse spontaneous uterine motility is generated earlier in gestation than in the rabbit. The activity seen in the myometrium of the mouse during the middle stages of gestation resembles that which appears in the rabbit uterus in the last stages of gestation. The rhythm, magnitude and frequency of the contractions and the spike discharges are less regular during pregnancy than post-partum, and contractions during the whole of gestation are less well synchronized with the electrical spikes than post-partum. In the rabbit, the sensitivity to oxytocin is 1000 times greater post-partum than during pregnancy (Kuriyama & Csapo, 1959*b*) but in mice the sensitivity of the myometrium to oxytocin does not alter.

Values of the membrane potential in the mouse myometrium varied considerably; this may be due to the small diameter of the fibres and their great variation in size. The mouse myometrium had a higher membrane potential in pregnancy than post-partum. This was also observed in the rabbit by Goto & Csapo (1957) and in the rat by Kuriyama & Csapo (1961*b*). Furthermore, the membrane potential was higher in the placental region than at non-placental sites in the rabbit (Goto & Csapo, 1959), in the rat (Thiersch *et al.* 1959; Kuriyama & Csapo, 1961*b*) and in the mouse (Goto *et al.* 1959). In general, the measured membrane potential in the myometrium was always lower than the theoretical potential calculated from the ratio of K concentrations inside and outside the cell (83 mV by Horvath,

1954; 81 mV by Daniel, 1958). Also, from measurements of internal and external Na concentrations a theoretical overshoot of 13 mV would be predicted (Daniel, 1958, cf. Woodbury & McIntyre, 1954), but the recorded action potentials rarely showed an overshoot. This discrepancy between theoretical and observed values may be due to the effect of hormones on the membrane structure, changing its permeability to Na^+ and Cl^- (Kuriyama, 1961).

The differences found between pregnant and post-partum mouse myometria in frequency and magnitude of train discharges and in the number of spikes in a train are similar to those in the rabbit and rat (Kuriyama & Csapo, 1959*b*, 1961*b*).

Treatment of late-pregnant and post-partum mouse myometria with progesterone *in vitro* decreased the number and size of action potentials and tension developed. The most striking effect was the block of propagation of the spikes, resulting finally in a train discharge of electrotonic (or local) potentials only, from which no spikes arose. This may be due to a decreased excitability of the membrane. As the number of spikes decreased, myometrial tension declined. Since the electrical recordings were made from single cells impaled with a micropipette, whereas the tension was recorded from the whole tissue, no definite conclusion can be drawn, but it is likely that the weakening of contractions is due to a decrease in the number of cells involved.

The pregnant and post-partum myometria did not differ qualitatively in their responses to progesterone. During pregnancy progesterone took longer to inhibit contraction and to cause hyperpolarization, and the degree of hyperpolarization produced was less.

Calcium-deficient solutions also blocked contraction in the mouse myometrium, as did progesterone, and the blocking action developed more slowly in the late-pregnant mouse uterus than post-partum. Coutinho & Csapo (1959) suggested that in pregnancy calcium ions might be more strongly bound to the tissue than in the post-partum myometrium and that therefore calcium-deficient solutions cause depolarization of the membrane more quickly in post-partum than in pregnant myometria.

It may be that calcium ions react with progesterone in the membrane of the myometrium. The above experimental results indicate that this interaction of progesterone with calcium ions in the membrane may contribute to the inactivation of the sodium carrier (Frankenhauser & Hodgkin, 1957), thus decreasing membrane excitability (and increasing membrane stability). This may be one of the main causes of the block of propagation. There are, however, no direct observations that progesterone reacts in the membrane with calcium as a chelating compound. On the other hand, progesterone is a redox reagent and an accelerator of tissue metabolism, and therefore it might act by increasing phosphorylation, thereby supplying energy for changes of ionic conductance including the 'stabilization' mechanism in the membrane. This might lead to hyperpolarization and to an increase of the threshold of excitation of the membrane. Such a mechanism has been suggested for the inhibitory action of adrenaline in the taenia coli (Axelsson, Bueding & Bülbring, 1959; Axelsson & Bülbring, 1960). While oxytocin increased the magnitude and frequency of contraction but

had no effect on the irregular periodic contractions which often appear in the post-partum myometrium, these irregularities were abolished by progesterone, and after progesterone was washed out the muscle showed regular contractions for long periods.

In conclusion, the experiments in this paper suggest that progesterone increases the stability of the myometrial membrane, raises the threshold of excitation and blocks the propagation of activity.

SUMMARY

1. The myometrium from mice in the middle and late stages of pregnancy and post-partum were used to record tension and membrane activity.

2. Progesterone abolished the contractions. The time taken for the abolition and for the return to the former magnitude after washing was longer in late-pregnant than in post-partum myometria.

3. Calcium-deficient solutions had an action on isometric contraction very similar to that of progesterone in both conditions.

4. When the myometrium was treated with progesterone and oxytocin together, the two substances had at first an additive effect in increasing the frequency of contractions and the resting tension, but then progesterone dominated and contractions were abolished.

5. The mean value of the membrane potential was higher in late-pregnant than in post-partum myometria. In the mouse, unlike other species, the membrane remained continuously depolarized during a train of discharges. The variation in the values of membrane potential, spike size, number of spikes in a train discharge, and duration of the train discharges was less in post-partum than in pregnant myometria.

6. The myometrial action potentials of mice in the middle and the late stages of pregnancy were irregular, and the contractions of the muscle were not well synchronized with the action potentials recorded from single cells.

7. When the mouse myometrium was stretched to 50, 75, 100, 125 and 150 % of its resting length, an increase in membrane activity was observed with depolarization and an increased number of spikes in a train.

8. Oxytocin caused depolarization, and increased the number of train discharges as well as the number and frequency of spikes in a train.

9. Progesterone decreased the number of spikes in a train and blocked propagation. After the cessation of spontaneous activity the membrane became hyperpolarized.

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THE ROLE OF CALCIUM IN THE SECRETORY RESPONSE OF THE ADRENAL MEDULLA TO ACETYLCHOLINE

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It is commonly believed that acetylcholine (ACh) is the physiological transmitter of sympathetic nerve impulses at the adrenal medulla. The reasons are the following: the sympathetic nerve fibres that innervate the adrenal medullary cells are developmental homologues of the preganglionic fibres elsewhere in the sympathetic nervous system which are known to be cholinergic; on stimulation of the adrenal nerves an acetylcholine-like substance is released from the adrenal gland (Feldberg, Minz & Tsudzimura, 1934); ACh is a powerful stimulant of adrenal medullary secretion; and finally, transmission at the adrenal medulla is affected by a variety of drugs in much the same way as these drugs affect transmission at the established sites of cholinergic transmission at sympathetic ganglionic synapses.

The purpose of the present experiments was to study the mechanism by which ACh brings about adrenal medullary secretion and, as a first approach, to observe how the response of the gland to ACh might be influenced by changes in the ionic composition of the extracellular environment. Our experiments show that the excitant action of ACh on the adrenal medulla is dependent on the presence of calcium, and suggest that ACh evokes adrenal medullary secretion by causing calcium ions to penetrate the adrenal medullary cells.

Short accounts of these experiments were presented at the Oxford meeting of the Physiological Society and at the First International Pharmacological Meeting earlier this year (Douglas & Robin, 1961*a*, *b*).

METHODS

All experiments were done on cats anaesthetized with chloralose (90 mg/kg I.V.) following ethyl chloride and ether. A tracheal cannula was inserted and artificial ventilation begun with minute volume sufficient just to suppress spontaneous respiratory movements. This was done to prevent any adrenal discharge which would otherwise have resulted from failure of ventilation during the later operative procedures which led to pneumothorax. A mid line abdominal incision was made from the xiphisternum to the pubis and the entire gastro-intestinal tract removed. The xiphisternum and lower ribs were retracted headwards to provide free access to the adrenal glands. One or both adrenal glands were then prepared

for perfusion. In some experiments one or both glands were perfused *in situ*; more often, however, one or other adrenal gland was removed from the animal and perfused *in vitro*.

Perfusion of the adrenal glands in situ. In a number of preliminary anatomical studies casts were made of the arterial supply to the adrenal glands by injecting thin liquid plaster of Paris under considerable pressure through a cannula in the abdominal aorta. By this means it was shown that the arterial supply was both complex and variable. Arterial twigs to the adrenal gland were seen coming from: (1) the adrenolumbar arteries and the phrenic artery, (2) the coeliac axis and superior mesenteric artery at various distances from their origins from the aorta, (3) the renal arteries at varying positions between the abdominal aorta and kidneys, and (4) the abdominal aorta (especially its ventral aspect) at varying distances caudal from the adrenals as far as, or even below, the origin of the renal arteries. Gross variation from animal to animal was apparent and it was obvious that any *close* arterial perfusion was impracticable. A more remote perfusion technique was therefore adopted as follows:

(1) A series of ligatures was placed around the border of the right kidney, and the renal vein and artery tied off close to the hilus.

(2) The right kidney was elevated and the adrenolumbar artery and vein tied off just lateral to the right adrenal gland.

(3) The diaphragmatic branches of the right adrenolumbar and coeliac arteries were tied along with any associated veins (the coeliac axis and superior mesenteric arteries had been tied and cut during evisceration). Neighbouring vascular shunts were occluded by cutting the right crus of the diaphragm between ligatures.

(4) The right greater and lesser splanchnic nerves were transected.

(5) While the right kidney was reflected medially (but not so as to interfere with the circulation of the right adrenal), ligatures were placed around the three or more pairs of perforating lumbar arteries which arose from the abdominal aorta on its dorsal aspect in the region to be perfused.

(6) The dorsal aspect of the aorta was completely freed from the prevertebral tissue to interrupt sympathetic nerve fibres that connect the adrenals and the sympathetic chain in this region (Vogt, 1952).

(7) Steps 1–4 were repeated on the left side.

(8) The aorta was tied immediately above the coeliac axis and perfusion begun through a glass cannula inserted into it about 1·5 cm below the renal artery and pointing headwards; this cannula was fitted with a bubble trap.

(9) The inferior vena cava was tied below the liver and above the entry of the right adrenolumbar vein. Perfusate was collected through a polythene cannula inserted into the vena cava at the same level as the arterial cannula.

(10) A third cannula, made of polythene tubing and fitted with a tap, was inserted into the coeliac axis. During the change over from one perfusion solution to another, fresh fluid was first washed vigorously through a side arm in the 'inflow' arterial cannula in the aorta, and then the tap of this third 'outflow' cannula was opened and the remaining dead space in the abdominal aorta washed out. In this way the effective dead space in the system was equivalent to the small volume of fluid in the fine arterial twigs supplying the gland.

(11) As soon as perfusion was begun the animal was killed by cutting open the thoracic aorta so that arterial pressure fell to zero and no blood could thereafter find its way into the perfused region through any untied anastomosis.

The gland was perfused at a constant pressure of about 70 mm Hg provided by a large reservoir of compressed air. Flow through the perfused region was about 3–4 ml./min. The total blood flow through both the cat's adrenals has been estimated to be approximately 1–3 ml./min (Stewart & Rogoff, 1917).

Perfusion of the isolated adrenal gland. During the course of the investigation it was found

more convenient for many purposes to isolate a single gland, either the right or the left, and perfuse it in the reverse direction through the adrenolumbar vein. This procedure had the advantage of limiting the perfusion to the adrenal tissue and thereby allowing a higher concentration of catecholamines in the perfusate. This was of especial value when, for example, the perfusate contained excessive amounts of potassium, which would tend, in large volume, to interfere with the bioassay. The preparation had the additional advantage of being comparatively simple to set up.

The procedure was as follows: The animal was eviscerated as previously described, and one or other kidney retracted to reveal the corresponding adrenolumbar vein. Any tributaries from extra-adrenal sources which joined this vein in its course across the gland to the inferior vena cava were tied and a fine polythene cannula connected to the perfusion system was tied into the vein lateral to the gland and pointing toward it. The adrenolumbar vein was then tied close to the vena cava and retrograde perfusion begun with a pressure of about 70 mm Hg. The thoracic aorta was then cut open to reduce systemic blood pressure to zero, and the adrenal gland cut free from the animal as rapidly as possible so that it would not be stimulated by discharges in the sympathetic nerves set up by the terminal asphyxia. The gland, attached to the end of the perfusion cannula, was transferred to a filter funnel filled with liquid paraffin. The effluent from the cut ends of the many small arterial twigs accumulated in the stem of the funnel and could be drawn off at appropriate intervals.

Perfusion fluids. Perfusion was usually carried out with phosphate-buffered Locke's solution of the following composition (mM): NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85; glucose 10. When equilibrated with pure oxygen this solution had a pH close to 7·0. Similar solutions were used deficient in sodium, potassium or calcium. When sodium was removed the osmotic pressure of the solution was restored with sucrose (0·9 % NaCl was considered to be isosmotic with 9·25 % sucrose). Some solutions contained 5 or 10 times the normal amount of potassium (28 or 56 mM). In these solutions sodium was reduced by a corresponding amount. In some experiments to test the effect of various calcium concentrations bicarbonate-buffered Locke's solution of the following composition was used (mM): NaCl, 154; KCl, 5·6; $CaCl_2$, 0·5, 2·2, 8·8 or 17·6; $NaHCO_3$, 6; glucose 10. These solutions were equilibrated with 5 % CO_2 in O_2 and had a pH close to 7·0. In some experiments the perfusion fluid was pure sucrose in a strength (9·75 g/100 ml.) isosmotic with Locke's solution. Sometimes calcium (2·2 mM) was added to this sucrose solution. ACh chloride was added to any of these solutions when necessary in a concentration of 10^{-5} g/ml. All solutions contained ascorbic acid 10^{-5} g/ml. to inhibit oxidation of catecholamines. Perfusion was carried out at room temperature (24–26° C). Samples of perfusate were collected into flasks kept on ice, and then immediately frozen for subsequent assay.

It should be noted that we have observed no consistent or significant effect of ACh on the flow of perfusate through the adrenal. In some experiments the flow in the presence of ACh was identical to that in its absence: in other experiments the flow during perfusion with ACh was slightly above or below the levels found in ACh-free Locke's solution, but the effect was no greater than that which tended to occur spontaneously and which had no detectable influence on catecholamine output.

Assay of catecholamines. In response to electrical stimulation of the splanchnic nerves, or to ACh or potassium, the adrenal glands release the two catecholamines noradrenaline and adrenaline. The relative amounts vary widely, not only in different experimental conditions, but in apparently similar experimental conditions in different investigations (von Euler, 1956). Since our immediate interest was the total catecholamine output, the test object chosen for assay was the rabbit's thoracic aorta, which according to Furchgott & Bhadrakom (1953) is only very slightly more sensitive to noradrenaline than to adrenaline. The preparation was made as described by Furchgott (1960), and suspended at 37° C in Krebs's solution of the following composition (mM): NaCl, 118; KCl, 4·73; CaCl, 2·54; KH_2PO_4, 1·18; $NaHCO_3$, 24·9; glucose 11·09. This was equilibrated with 5 % CO_2 in oxygen and contained

the disodium salt of EDTA (ethylenediaminetetra-acetic acid) (10^{-5} g/ml.) to chelate heavy-metal ions and slow oxidation of the catecholamines (Furchgott, 1960). Such a preparation almost always gave satisfactory contractions when 0·02–0·03 μg of adrenaline was added to the bath (8 ml.). A number of preparations were about ten times as sensitive. There is ample evidence that the smooth-muscle stimulating substances released from the adrenal gland by splanchnic stimulation, ACh or potassium are adrenaline and noradrenaline. The active material in perfusates obtained in the present experiments caused contractions identical with those caused by adrenaline and was blocked to the same extent as adrenaline by partially blocking doses of the blocking drug phentolamine (Regitine). Assays were usually performed with doses equivalent to 0·05 μg or less of adrenaline (the values were expressed in terms of adrenaline bitartrate). At such levels discrimination between different doses was good and recovery prompt, so that tests could be made at intervals of a few minutes. It was found that by careful and repeated bracketing of test and standard samples an estimate of the adrenaline content of the 'test' sample could be expected not to deviate by more than about 10% from the true value.

Recording of contractions. Shortening of the aortic strip was recorded by an isotonic lever with a load on the tissue of 4 g. The small doses of catecholamines used caused an extremely small shortening of the aortic strip (about 1% of its length) and, with the 4 cm length we employed, required a friction-free and sensitive recording apparatus. Movement of the lever was therefore measured, amplified and recorded electrically as follows: A metal rod was suspended from the lever in such a way as to act as a moving core of a coil carrying alternating current. Changes in the position of the lever thus caused changes in the inductance of the coil. By incorporating this coil in a bridge circuit 'out of balance' currents were obtained which were amplified and used to drive an ink recorder. The over-all 'mechanical plus electrical' gain of the system was usually chosen to be about $\times 50$.

RESULTS

Effect of ACh in Locke's solution

Some preliminary experiments were carried out to establish appropriate conditions for stimulation by ACh. It was found that during perfusion with Locke's solution there was always some spontaneous output of catecholamines. The amount varied in different experiments, but was most commonly about 0·05–0·1 μg/min/gland (at the beginning of perfusion, the value was occasionally several times higher). Switching from Locke's solution to Locke's solution plus ACh 10^{-5} g/ml. always caused a large increase in catecholamine secretion. In the first 2 min the rate not uncommonly rose to 15 μg/min/gland; thereafter, as perfusion with ACh was continued the output fell to reach about 10% of this rate by about the tenth minute (Fig. 1). The reasons for this falling output have not been explored. For the present purposes it was found satisfactory to make tests of the adrenal response to ACh lasting 30 sec or 4 min, and to repeat these tests at intervals of about 20 min. Although there was some fall off in the amount of catecholamines released in successive tests, the responses were maintained sufficiently well to permit experiments of the sort we performed (viz. Fig. 4).

The first group of experiments was designed to examine the effect of a

reduction in the extracellular concentration of one or other of the cations sodium, potassium, or calcium upon the catecholamine-releasing effect of ACh. The procedure adopted in these experiments is typified by the following experiment with K-free Locke's solution.

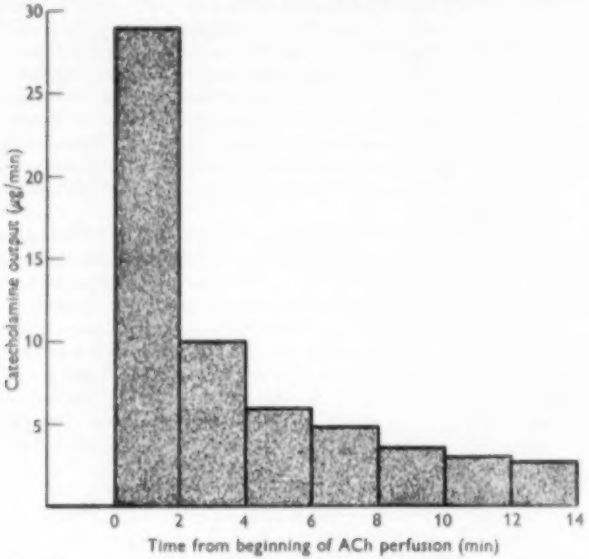

Fig. 1. The time course of catecholamine release during continuous perfusion of both adrenal glands with ACh. After a control period of perfusion with Locke's solution, during which catecholamine output was 0·064 μg/min (indicated by the thickening of the base line) perfusion was changed to Locke's solution containing ACh 10^{-5} g/ml. and successive 2 min samples taken.

Effect of ACh in K-free Locke's solution

In the experiment of Fig. 2*a*, an adrenal gland was isolated from the body and perfused *in vitro* with Locke's solution. After 16 min, during which four 'resting' samples of perfusate were collected for assay, perfusion was switched rapidly to a second solution identical with the first except that it contained ACh in a concentration of 10^{-5} g/ml. Perfusion with this ACh-containing medium was continued for 4 min and the perfusate was again collected for assay. Immediately following collection of this sample perfusion was changed to K-free Locke's solution. At the end of a further 16 min and collection of four more 'resting' samples, perfusion was again switched, this time to K-free Locke's solution containing ACh (10^{-5} g/ml.) and another 4 min sample obtained. Then the whole sequence was repeated several times to observe further the stimulant effect of ACh in Locke's

solution and in K-free Locke's solution. All the samples were assayed and the output of catecholamines per minute during each collection period was calculated. As may be seen from Fig. 2*a*, which shows a typical series of responses obtained in this way, perfusion with ACh resulted in a huge outpouring of catecholamines amounting to several micrograms per minute. The absence of potassium from the perfusion fluid did not diminish the excitant effect of ACh on catecholamine release: on the contrary, in each of a number of tests it enhanced the effect.

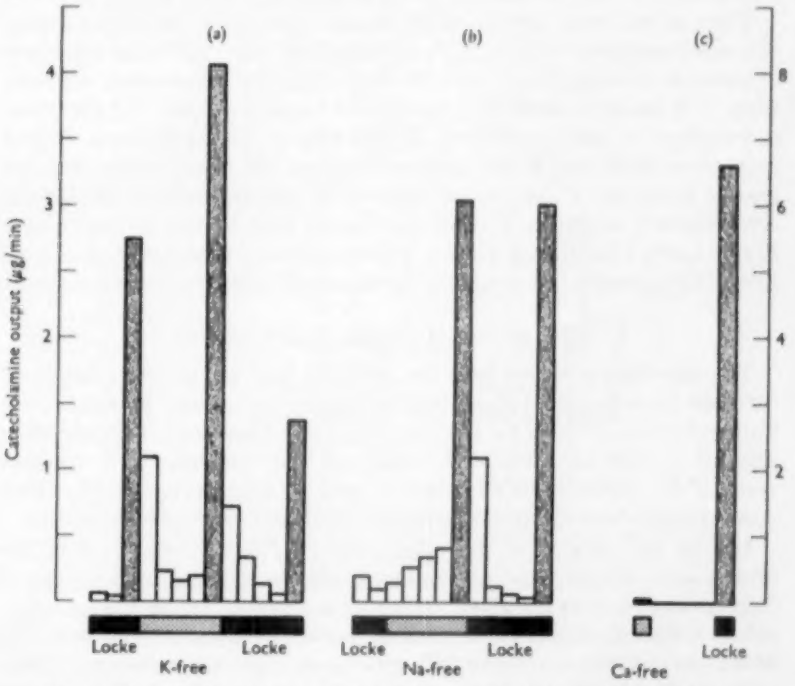

Fig. 2. The effect of omission of various cations on the amount of catecholamine released from the adrenal gland by ACh. The three experiments (*a*), (*b*) and (*c*) were performed on different adrenal glands perfused through the adrenolumbar vein with Locke's solution free from KCl (*a*), NaCl (*b*) or $CaCl_2$ (*c*). The deficient solutions are indicated by the stippled horizontal bars. Catecholamine output was measured in successive 4 min collection periods in each experiment. The solid vertical bars show the output in response to the addition of ACh 10^{-6} g/ml. during a 4 min period. The open vertical bars show the 'spontaneous' outputs of catecholamines. The scale on the left applies to (*a*): the scale on the right applies to (*b*) and (*c*). Since changing from Ca-free Locke's solution to Locke's solution caused a transient secretion (see Fig. 3), for clarity 'spontaneous' outputs have been omitted from (*c*).

Effect of ACh in Na-deficient Locke's solution

Similar experiments were done to test the effect of ACh during perfusion with Na-deficient Locke's solution in which NaCl was replaced with an isosmotically equivalent amount of sucrose. The solution was otherwise identical with the control Locke's solution and like it contained a small amount of sodium in the form of phosphate buffer. In these experiments a large output of catecholamines was still evoked by ACh after 16 min perfusion with the Na-deficient medium (Fig. 2*b*).

When several such periods of Na deprivation were interpolated during prolonged perfusion with Locke's solution, the outputs of catecholamines in response to stimulation by ACh were strikingly diminished, not only when ACh was administered in the Na-free Locke's solution but also when it was given in Locke's solution. In this respect the experiments differed from those involving K-free perfusion, where the deterioration was not greatly in excess of that which occurred in glands perfused throughout with Locke's solution. It was also noted that unlike perfusion with K-free Locke's solution, perfusion with a reduced concentration of sodium caused a progressive increase in the 'spontaneous' output of catecholamines.

Effect of ACh in Ca-free Locke's solution

The omission of calcium from the perfusion fluid had an effect strikingly different from the effect of omitting sodium or potassium. Perfusion with Ca-free Locke's solution for 16 min reduced the amount of catecholamines released by ACh to a very small fraction (5 % to less than 1 % in different tests) of the control value obtained in Locke's solution (Fig. 2*c*). The effect was reversible and could be repeatedly obtained in a single preparation.

During the periods of perfusion with Ca-free Locke's solution the spontaneous output of catecholamines tended to fall, and the total loss of catecholamines from the gland during such a period, before the introduction of ACh in Ca-free Locke's solution, was a small fraction (less than 5 %) of the outputs in response to ACh given earlier or later in the experiment while perfusing with Locke's solution. Clearly there was no significant depletion of catecholamines at the time when ACh, in Ca-free Locke's solution, failed to elicit secretion.

Release of catecholamines by calcium

It was found that the effect of calcium deprivation was not confined to suppression of the response to ACh. After a period of perfusion with Ca-free Locke's solution, some change occurred in the gland such that large amounts of catecholamines were released merely by reintroducing Locke's solution containing the normal amount of calcium (Fig. 3*a*). It does not

follow from such an experiment, however, that this effect of reintroducing calcium is due to an action on the adrenal medullary cells directly. The effect could be on the splanchnic nerve endings, since Hutter & Kostial (1955) found during experiments on the superior cervical ganglion perfused with Locke's solution that the amount of ACh released by nerve stimulation was occasionally enhanced if the test were preceded by a period of perfusion with Ca-free Locke's solution. If in our experiments there

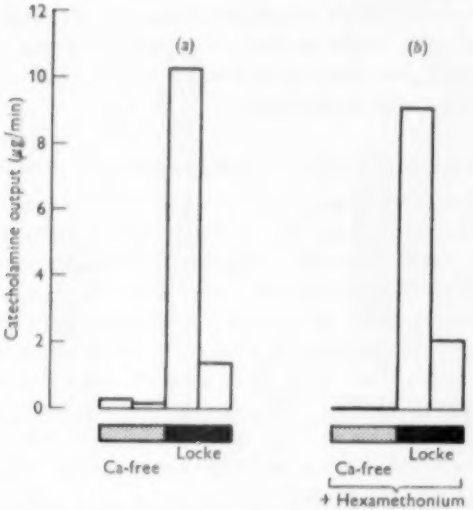

Fig. 3. Release of catecholamines by Locke's solution (indicated by solid horizontal bar) following perfusion with Ca-free Locke's solution (indicated by stippled horizontal bar). (*a*), both adrenals perfused *in situ*, (*b*), left adrenal perfused *in vitro*: hexamethonium (5×10^{-4} g/ml.) present throughout this experiment.

were any random activity in the sympathetic nerves, such as injury discharge, then reintroduction of calcium might increase the amount of ACh released and thereby increase catecholamine output. It is highly improbable, however, that a presumably small and random effect could account for a release of catecholamines of the magnitude found in our experiments. The amounts of catecholamines released by reintroduction of Locke's solution were usually several times greater than those we were able to release by maximal stimulation of the splanchnic nerves in Locke's solution. The possibility of such an indirect mechanism of catecholamine release was excluded by experiments with hexamethonium. This substance is known to suppress the secretory response to splanchnic nerve stimulation (Paton & Zaimis, 1952), and we have found that under our conditions

hexamethonium (5×10^{-4} g/ml.) reduces the response to maximal splanchnic nerve stimulation from about 2·5 μg/min to about a tenth of this value (this effect is illustrated in Fig. 5). As is seen from Fig. 3*b*, hexamethonium in this concentration did not prevent the reintroduction of calcium from causing its customary powerful effect and raising secretion to about 9 μg/min.

The finding that calcium in a normal concentration could stimulate adrenal medullary secretion in these abnormal conditions prompted us to test whether *excess* calcium would stimulate the gland during perfusion with Locke's solution. When perfusion was switched from Locke's solution to Locke's solution plus calcium 20 mM (i.e. to a total of 22·2 mM Ca) there was no obvious increase in secretion.

Quantitative relation between secretion and calcium concentration

It was found that there was a quantitative relation between the amount of catecholamines released by ACh and the concentration of calcium in the perfusion fluid over a wide range. This was shown by experiments in which responses to ACh (10^{-5} g/ml.) were tested alternately in Locke's solution and in a solution otherwise identical but containing more, or less, calcium. One of eight such experiments is illustrated in Fig. 4, which shows that catecholamine output was more than doubled when the calcium concentration was raised to 17·6 mM. This was the largest effect observed: higher concentrations of calcium were not studied. With 8·8 mM Ca the response to ACh was about 50% greater than the control value, while with 0·5 mM Ca it was about half the control value.

The effect of Ca lack on the response to potassium

It is not improbable that ACh may cause catecholamine secretion by depolarizing adrenal medullary cells, for it depolarizes the homologous sympathetic ganglion cells (Paton & Perry, 1953). Moreover, excess potassium (which may be assumed to depolarize) is known to release catecholamines from the medulla. It was therefore of interest to see whether the response to potassium was also dependent on the presence of calcium. The addition of potassium (50 mM) to Locke's solution caused the gland to secrete at a rate of 10 μg/min or more. This same concentration of potassium, however, was almost without effect when added to Ca-free Locke's solution at the end of 16 min perfusion with this medium. Although it is known that potassium excites adrenal medullary cells directly (Vogt, 1952), experiments were done with hexamethonium to rule out the possibility that the effect of potassium observed in our experiments was indirect and the consequence of stimulation of splanchnic nerve endings. One such

experiment is illustrated in Fig. 5, which shows that potassium evoked a powerful secretion under conditions in which the response to maximal splanchnic stimulation was enfeebled by hexamethonium, and also that potassium failed to release catecholamines when calcium was absent.

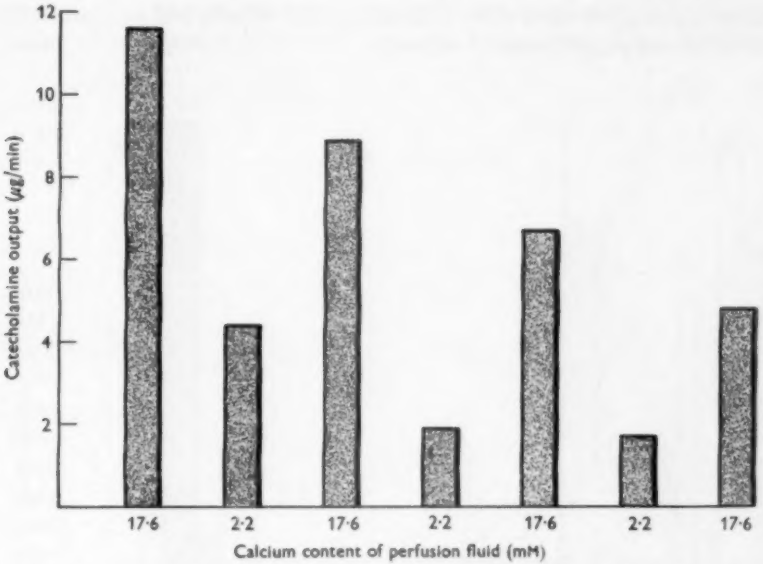

Fig. 4. The potentiating effect of excess calcium on the amount of catecholamine released by ACh. An adrenal gland was perfused *in vitro* alternately for 16 min periods with Locke's solution (2·2 mM Ca) and a 'high-calcium' Locke's solution (17·6 mM Ca). During the last 4 min of each period of perfusion with one or other solution, ACh 10^{-5} g/ml. was introduced for 30 sec. The vertical bars show the secretory responses obtained sequentially in this way.

Perfusion with sucrose plus calcium

Some experiments were done to see whether calcium alone, in the absence of sodium or potassium, might be sufficient to allow ACh to release catecholamines. Perfusion was carried out with isotonic sucrose containing calcium 2·2 mM and a small amount of sodium phosphate buffer (1·3 mM). A complication arose in that this Ca-sucrose solution itself caused a steadily mounting output of catecholamines reaching a level of several micrograms per minute. In such experiments (Fig. 6*a*) ACh failed to release further amounts of catecholamines, although the rate of secretion was usually well below that which might have been expected from ACh or the rate that could be achieved in different circumstances (e.g. Fig. 6*c*).

The strongly stimulant effect of the Ca-sucrose medium could not be attributed to the pH, for although acidity is known to cause release of catecholamines (von Euler & Stjärne, 1955) the solution was buffered to the same pH as the Locke's solution. Apparently the effect resulted from the withdrawal of sodium and potassium together. As we have described earlier, removal of one or other of these ions individually had comparatively minor effects on spontaneous release.

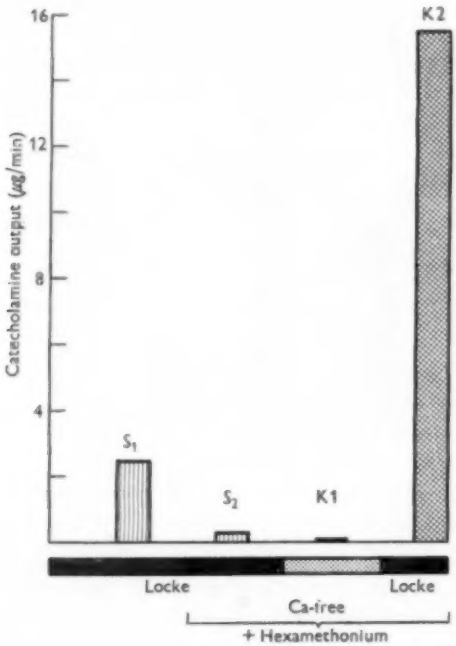

Fig. 5. An experiment on an adrenal gland perfused *in vivo* showing that potassium evokes medullary secretion in the presence of hexamethonium sufficient to suppress the stimulant effect of splanchnic nerve stimulation and that this effect depends on calcium.

The four responses were obtained sequentially at intervals of about 20 min. *S* 1 and *S* 2 show the catecholamine outputs to 4 min periods of maximal stimulation of the splanchnic nerve at 10 shocks/sec before and after the addition of hexamethonium 5×10^{-4} g/ml. to the perfusion fluid (Locke's solution). K 1 and K 2 show the catecholamine outputs to 4 min periods of perfusion with potassium (56 mM) in Ca-free Locke's solution and Locke's solution, each containing hexamethonium 5×10^{-4} g/ml. (Tonicity of the 'high-potassium' solutions was maintained by appropriate reduction of sodium.)

Perfusion with pure isosmotic sucrose

Perfusion with pure isosmotic sucrose free from calcium was also found to cause catecholamine release. This effect was seen not only when this medium was introduced after Locke's solution and when some calcium would still be present, but also in experiments in which Ca-free sucrose was given after a period of 16 min perfusion with Ca-free Locke's solution

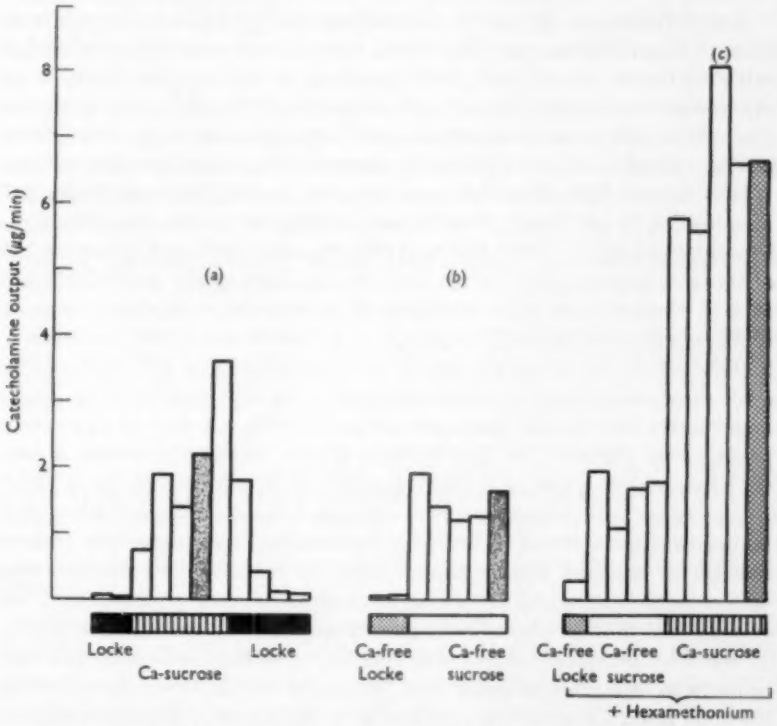

Fig. 6. Experiments on three adrenal glands perfused *in vitro* showing stimulation of catecholamine output by isosmotic sucrose with or without calcium, and in the presence or absence of hexamethonium. Catecholamine outputs in successive 4 min collection periods are shown. (*a*), the effect of sucrose plus 2·2 mM calcium following Locke's solution. (*b*), the effect of Ca-free sucrose following Ca-free Locke's solution. (*c*), the effect of adding calcium to Ca-free sucrose (hexamethonium 5×10^{-4} g/ml. present throughout *c*).

ACh 10^{-5} g/ml. was present during the periods indicated by the solid vertical bars in (*a*) and (*b*). K (56 mM) was present during the period indicated by the cross-hatched vertical bar in (*c*) (isotonicity was maintained by appropriate reduction of sucrose).

(Fig. 6*b*). Such effects of Ca-free sucrose were also seen in glands perfused with hexamethonium (Fig. 6*c*) and must therefore involve the medullary cells directly. When calcium was added during such perfusion the output of catecholamines increased greatly, but it was not further increased by the addition of potassium (56 mM) to the sucrose medium (Fig. 6*c*).

DISCUSSION

Acetylcholine clearly evokes adrenal medullary secretion through some calcium-dependent process. This seems to involve the release or ejection of catecholamines rather than their synthesis or storage, for there is no significant loss of catecholamines from the gland during Ca-free perfusion when ACh fails to cause secretion, and large amounts of catecholamines can be released in such conditions by other stimuli such as isosmotic sucrose.

It is known that calcium has an important action in maintaining cell membranes, in particular their impermeability to various ions (Frankenhaeuser & Hodgkin, 1957; Harris, 1960; Maizels, 1960) and a passive role of this sort might explain *some* of our results, such as the reduced efficacy of ACh when calcium is low and its lack of effect when calcium is absent. It offers, however, no ready explanation for the findings that the response to ACh is potentiated by calcium in high concentrations and that calcium itself may cause secretion. These suggest to us that calcium may play a more active role in the stimulant action of ACh. A clue to this role is provided by the evidence that calcium is only effective in releasing catecholamines after a period of calcium deprivation, and that as much as ten times the normal concentration of calcium fails to evoke secretion when added during perfusion with Locke's solution containing the normal amount of calcium. The stimulant effect of reintroducing calcium thus occurs in circumstances known to increase membrane permeability. The effect could thus be due to calcium ions penetrating the medullary cells. If the level of ionized calcium within the medullary cells is as low as it appears to be in various other cells (Keynes & Lewis, 1956; Harris, 1957), then an inward flux of calcium ions is to be expected if permeability to them is increased.

The possibility thus arises that ACh may cause secretion by acting on the membranes of the adrenal medullary cells to cause them to take up calcium ions. This does not seem unlikely. At each of the sites of cholinergic transmission where the role of ACh has been rigorously examined (e.g. the motor end-plate, del Castillo & Katz, 1956) it appears to be confined to the membrane, probably its outer surface, and to involve the production of some increase in membrane permeability to common species of ions. There is, moreover, evidence that ACh does not act directly on the intracellular

stores of catecholamines (the adrenal medullary granules) to cause release of catecholamines (Blaschko, Hagen & Welch, 1955; Schümann & Weigmann, 1960). If ACh acts on medullary cells to increase their permeability to calcium, an inward movement of calcium ions would be expected for the reasons we have just given. Such an influx of calcium ions in response to ACh has already been found in muscles (Robertson, 1960; Shanes, 1961). It would also be expected that the amount of calcium ions passing inward would depend on the extracellular calcium-ion concentration, as has again been found in muscle (Holland & Sekul, 1959). Such an active role of calcium in the secretory response of the adrenal medulla to ACh would account not only for the depressant effects of low and zero calcium concentrations, but also for the potentiating effects of calcium concentrations above the normal. It would also be consistent with the finding that omission of potassium enhances the secretory response to ACh, for calcium influx into muscles is known to be enhanced in such circumstances (Niedergerke & Harris, 1957; Holland & Sekul, 1959).

The fact that isosmotic sucrose liberates catecholamines in the absence of extracellular calcium does not argue against the hypothesis that calcium links the ACh stimulus with the secretory response, but indicates that catecholamines may also be released by other mechanisms. Whether it is the sucrose, or the lack of sodium and potassium, which is responsible for the release is uncertain, but the latter possibility is suggested by the fact that sucrose containing potassium 5·6 mM was much less effective. It is known that in pure sucrose the catecholamine granules isolated from the adrenal medullary cells tend to lose their amine content (Hillarp, 1958; Schümann & Weigmann, 1960), but this effect is rather small at the temperatures at which we worked, and moreover it seems improbable that significant quantities of sucrose penetrate the medullary cells. The effect of isosmotic sucrose on the whole gland seems most probably to be a membrane phenomenon. It is of interest that the almost complete replacement of sodium with sucrose, which we found causes a progressive increase in the 'resting' discharge of catecholamines, causes an increased uptake of calcium and its accumulation in heart muscles (Niedergerke & Harris, 1957).

Although calcium is obviously able to evoke catecholamine secretion in the whole gland, Hillarp (1958) has reported that the addition of calcium 1 mM does not increase the release of catecholamines from isolated adrenal medullary granules suspended in sucrose. Although this might simply mean that some other intracellular constituent is required along with calcium, there are grounds for believing that the process of secretion occurring in the whole cell does not involve the intracellular disruption of these granules or indeed the leakage of catecholamines from them. Rather, from electron-microscopic studies it seems that the process of adrenal medullary secretion involves a complex chain of events (referred to as 'vesiculation and membrane flow') during which the secretory granules are first attached to the membrane and their contents then extruded through it (De Robertis, Nowinski & Saez, 1960). This may be the process

which is calcium-dependent. Calcium is known to have an influence not only on cell membranes but also on the physico-chemical properties of cell sap (Heilbrunn, 1943, 1956; Hodgkin & Katz, 1949; Chambers & Kao, 1952).

It is not known if ACh depolarizes adrenal medullary cells but it acts in this way at other sites including the developmentally homologous ganglion cells (Paton & Perry, 1953). Since we have found that potassium in concentrations which presumably cause considerable depolarization also excites medullary secretion through some calcium-dependent process, and since depolarization is known to cause an influx in calcium ions in muscles and nerves (Bianchi & Shanes, 1959; Hodkgin & Keynes, 1957) it is not improbable that ACh could cause calcium influx at the medulla by depolarization. However, the omission of sodium from the perfusion fluid for 16 min did not seriously depress the response to ACh although it might be expected to impair depolarization due to sodium influx, and it is thus possible that ACh may stimulate calcium uptake without depolarization. (The loss of the stimulant response to ACh after more lengthy perfusion with sodium-free Locke's solution may be due to desensitization of ACh receptors such as appears to occur at motor end plates in such a medium (del Castillo & Katz, 1955).

Because of the common developmental origins of the adrenal medullary cells and the post-ganglionic sympathetic neurones, the present experiments obviously provide grounds for supposing that calcium may be similarly involved in the secretion of noradrenaline on the arrival of impulses at sympathetic nerve endings. In nerves which are cholinergic calcium appears to act in this way as a link between the nerve impulse and the secretion of ACh, and calcium penetration into the nerve ending during activity has been postulated as the trigger for ACh secretion (Birks & MacIntosh, 1957; Hodgkin & Keynes, 1957). Although the stimulus to secretion in nerves is the action potential while that to the medullary cell is ACh, a common mechanism—depolarization—may underly both responses. A further inference from our experiments is that other biologically active substances may be released from their intracellular stores by calcium entry. Calcium is required for the release of histamine and 5-hydroxytryptamine during the antigen–antibody reaction (Humphrey & Jaques, 1955) and the amount of histamine released varies with the calcium-ion concentration (Mongar & Schild, 1958).

Finally, each of the main pieces of evidence which together suggest to us that calcium somehow links the ACh stimulus to the secretory response in the adrenal medulla has a parallel in the evidence which has led others to propose that calcium influx links stimulation with muscular contraction (Heilbrunn, 1943, 1956; Sandow, 1952; Shanes, 1958). Thus, omission of

calcium leads to failure of contraction (Frank, 1960): force of contraction varies with extracellular calcium concentration (Niedergerke, 1956): calcium causes contraction provided the membrane barrier to it is removed (Podolsky & Hubert, 1961): and omission of potassium increases force of contraction (Niedergerke & Harris, 1957). There are obvious similarities between the process which Sandow has described as 'excitation-contraction coupling' and what we may term 'stimulus-secretion coupling'. The extent of the similarity and its significance remains to be discovered.

SUMMARY

1. Experiments have been made to determine how ACh, the chemical transmitter of sympathetic effects at the adrenal medulla, causes the medullary cells to secrete.

2. In perfused cat's adrenals a profound lowering of the sodium content of the perfusion medium for 16 min did not cause much reduction in the output of catecholamines in response to ACh. When potassium was omitted for a similar time the response to ACh was increased.

3. The omission of calcium, however, virtually abolished the secretory response of the adrenal medulla to ACh. The effect did not seem attributable to reduction of catecholamine stores and was considered to involve the process of catecholamine release.

4. Excess potassium (56 mM) also failed to have its usual powerful stimulant effect on catecholamine secretion when given during perfusion with Ca-free Locke's solution.

5. A quantitative relation was found between the amount of catecholamines released by ACh and the concentration of calcium in the perfusion fluid at values above as well as below the normal 2·2 mM Ca.

6. Calcium itself (2·2 mM) caused a vigorous discharge of catecholamines when introduced after a 16 min period of perfusion with Ca-free Locke's solution.

7. Isosmotic sucrose evoked release of catecholamines; addition of calcium greatly augmented this effect.

8. It is suggested that the role of ACh as a transmitter at the adrenal medulla is to cause some brief change in medullary cells which allows calcium ions to penetrate them and trigger the catecholamine ejection process.

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SOME *IN VITRO* OBSERVATIONS ON WATER, SODIUM AND POTASSIUM METABOLISM IN THE OESTROUS RAT UTERUS

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The electrolyte composition of the rat uterus is known to vary during the oestrous cycle and is influenced by the ovarian hormones (Cole, 1950; Kalman, 1957). The changes in electrolyte composition are accompanied by changes in contractility (see Ussing, 1960). The distribution of the electrolyte changes between the myometrium and endometrium of the rat is not clear, but in the rabbit, in which the two layers can be satisfactorily separated, changes occur in the smooth-muscle layers (Bitman, Cecil, Hawk & Sykes, 1959). It would thus seem possible that cyclical changes in electrolytes result in altered contractility. Smooth muscle differs from striated muscle in its electrical activity and its response to electrolytes in the solutions bathing it *in vitro* (see Davson, 1959). It has been suggested that the regulation of sodium and potassium content may also be different from that in striated muscle. Daniel & Bass (1956), for instance, have shown that *in vivo* changes of the extracellular sodium and potassium concentrations did not result in parallel changes in the two types of muscle. Bozler & Lavine (1958) found that frog stomach muscle swelled in isotonic sucrose solution, but shrank if calcium was present. They suggested that the muscle fibres were permeable to sucrose and did not behave as though they were surrounded by a semipermeable membrane, and that in the regulation of cell volume 'the charge of protein and intermolecular forces are involved'.

These observations suggested that it might be interesting to study weight changes, water content and sodium and potassium contents of the rat uterus *in vitro* under different conditions. As in this species the myometrium cannot be separated satisfactorily from the endometrium, these determinations were combined with histological examination of the tissues in an attempt to ascertain the distribution of the volume changes. The results include the effects of changes in tonicity and divalent ion concentrations as well as the effects of metabolic inhibitors, cardiac glycosides and oxytocin.

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METHODS

Animals. Young, but mature, albino virgin female rats (170–200 g) were injected intramuscularly with 0·1 mg of di-hydrostilboestrol (Oestrogenine; Fawns & McAllan) 48 hr and again 24 hr before use. They were killed by concussion and the uterus was dissected and placed in Krebs's solution.

Solutions. Pieces of uterus were normally bathed in the Medium III described by Krebs (1950). But the only energy substrate used was glucose, so that the final concentrations of the constituents were (mM): NaCl, 126·9; KCl, 5·37; $CaCl_2$, 2·87; $MgSO_4$, 1·43; $KHPO_4$, 1·34; $NaHCO_3$, 4·0; phosphate buffer 2·61 and glucose 13. The solution was gassed with a mixture of 95 % O_2 and 5 % CO_2 and the pH was 7·4. When an ion was excluded, the total osmotic concentration was maintained by the addition of sodium chloride. Analytical reagents were used throughout.

Experimental procedure. Each horn of the uterus was cut transversely into two pieces. A small platinum hook was inserted through one end of each of the four pieces of uterus so obtained. This hook was used to suspend the piece of uterus from the balance for weighing. The pieces of uterus ranged in weight from 110 to 140 mg. At the beginning of the experiment each piece was blotted on filter paper and weighed to the nearest 0·5 mg. It was then placed in a test tube containing 30 ml. of Krebs's solution, or a modification of it, which was kept in a water-bath at 37° C. At the end of the experiment the piece of uterus was weighed again on the hook and then by itself in a tared vitreosil test tube. In order to estimate the dry weight of the tissue the test tube containing the uterus was left in an oven at 105° C for 24 hr before being reweighed. The sample was then left overnight in a furnace at 550–580° C and the ash dissolved in distilled water (Cole, 1952; Desmedt, 1953). Sodium and potassium were determined with a flame photometer. In most cases a piece of uterus from the same animal was used for both the control and experimental observations. The experiments described in Tables 2 and 3 were all done in this way. In other experiments this was not always the case, and the statistical treatment of the data had to be altered accordingly.

Histology. Pieces of the uterus were fixed for sectioning in Bouin's solution and stained with haematoxylin and eosin. The mounted sections were photographed and the relative sizes of the cells in the different experiments determined by measuring the cell diameters in photomicrographs that had been magnified equally. This method shows up gross differences in cell size, as seen with solutions of differing tonicities or when calcium was absent from the bathing solution.

Drugs. Digoxin (Lanoxin; Burroughs, Wellcome and Co.); ouabain (Arnaud, Laboratoire Nativelle, Paris) and oxytocin (Syntocinon; Sandoz Products) were used.

Expression and interpretation of results. Robinson (1960) has pointed out that ideally in experiments with isolated tissues comparisons should be made of the same number of cells. A unit of either wet or dry weight is often assumed to represent a constant number of cells. But changes in the amount of tissue solids or in water content may upset this assumption, and when interpreting the results the units used must always be looked at critically. In the present experiments water content is expressed as kg/kg dry weight (Robinson, 1957; Heckman & Parsons, 1959). Sodium and potassium contents are given on a similar basis, i.e. as m-equiv/kg dry weight. It is obvious, however, that such an expression would be affected in the same way by a gain or loss of water, sodium or potassium as by a loss or gain of tissue solid. Electrolytes are therefore also expressed as m-equiv/kg tissue water. This expression will be independent of any loss of tissue solids but will be affected by changes in tissue water. Weight changes in incubated tissues have often been assumed to give indication of changes in cell volume by uptake or loss of fluid. An increase in weight no doubt reflects a fluid uptake, but the loss of high-molecular-weight tissue solids may well contribute substantially to a weight loss.

The oestrous rat uterus is spontaneously active in Krebs's solution. The various

experimental procedures may decrease (e.g. low potassium or calcium concentration) or increase (e.g. oxytocin) this activity for varying periods of time. It is not possible to predict with any accuracy how this will influence the results, as we do not know to what degree active transport processes can keep pace with the expected down-hill sodium and potassium movements across the cell membrane. The extracellular spaces of the tissues were not determined in the present experiments. As the extracellular contribution of sodium to the total cation content of the tissue is much greater than that of potassium, it will be more difficult to detect changes in sodium movement across the cell wall than those of potassium.

RESULTS

Incubation in normal Krebs's solution. The weight changes of pieces of uterus when incubated at 37° C in Krebs's solution are shown in Fig. 1. It can be seen that in contrast to liver and kidney tissue (Robinson, 1960) the pieces of uterus lost weight. If the tissue was incubated at 4° C for 3 hr this loss in weight was reduced, so that there was only a $1{\cdot}55 \pm 1{\cdot}04\,\%$ (S.E. of mean, 8 experiments) decrease in weight below the initial value.

The water, sodium and potassium contents of the pieces of uterus were measured after incubation in Krebs's solution (Table 1). The water content was unchanged after 1 hr but rose after 2 hr. There was a progressive increase in the amounts of sodium in the tissue and a smaller decrease in potassium. The (Na + K) content/kg water rose after 2 hr. If isotonicity with the bathing fluid is maintained, it would appear that an exchange of some sodium (and probably accompanying chloride) for tissue solids and potassium occurs.

Histological examination failed to reveal any changes in the size of the cells of the uterus under the above conditions.

Metabolic inhibitors. The presence of either 2 mM cyanide or 2 mM iodoacetate caused an increase in weight of the tissue (Table 2). In the case of iodoacetate the weight gain was associated with a gain in sodium and a smaller loss of potassium. With cyanide, no changes in electrolytes were detected.

Low potassium concentration. When the potassium concentration in Krebs's solution was reduced from 6·71 to 1·34 mM, a net gain of sodium and a smaller net loss of potassium occurred (Table 3). Histological examination showed a swelling of the cells under these conditions, especially in the muscle layers.

Absence of calcium. The pieces of uterus increased in weight when incubated in calcium-deficient Krebs's solution (Fig. 1). When magnesium was excluded, the uterus behaved as it did in normal Krebs's solution. Substitution of magnesium or strontium for calcium reduced but did not abolish the swelling. Incubation at 4° C instead of 37° C also reduced the swelling, so that after 3 hr it was only $4{\cdot}9 \pm 1{\cdot}28\,\%$ (8) of the initial weight. Histological examination of the tissues incubated in the various solutions

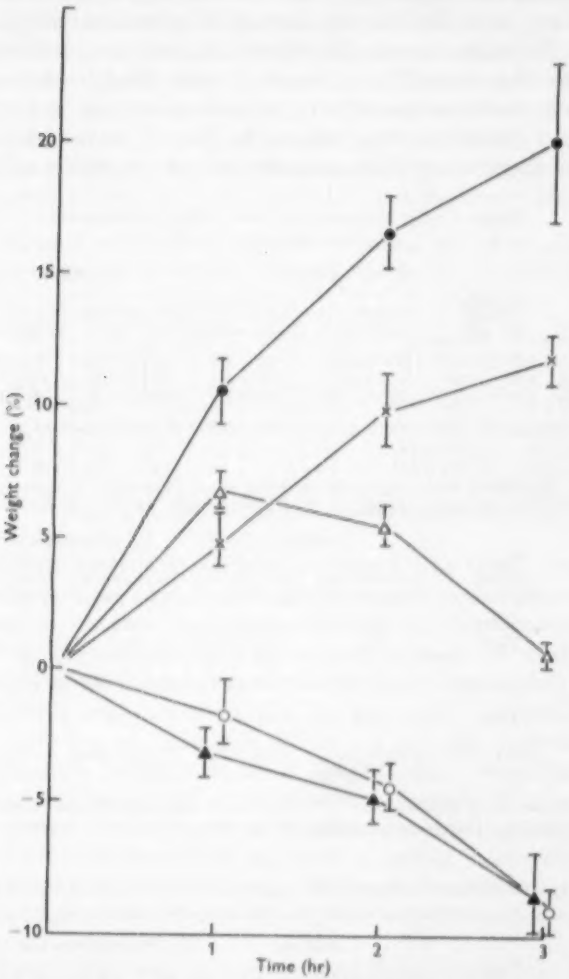

Fig. 1. Changes in weight of pieces of rat uterus incubated in normal and modified Krebs's solutions: ○ normal, ▲ no Mg^{2+}, ● no Ca^{2+}, △ Mg^{2+} substituted for Ca^{2+}, × Sr^{2+} substituted for Ca^{2+}. Each point on the graph represents the mean value of eight experiments and the vertical line indicates the standard error.

showed cellular swelling and this was most marked in the muscle layers. As can be seen from Table 4, the absence of calcium caused not only an increase in the water content of the tissue but also a drop in the content of both sodium and potassium/kg water, so that the (Na + K) content/kg water became much less than that in the bathing solution. On substitution of calcium by strontium or magnesium the (Na + K) content/kg water was normal, but there was an increase in the net gain of sodium and a smaller

TABLE 1. The effects of incubation of the rat uterus in Krebs's solution at 37° C

	Water (kg/kg dry wt.)	Sodium (m-equiv/kg dry wt.)	Sodium (m-equiv/kg tissue water)	Potassium (m-equiv/kg dry wt.)	Potassium (m-equiv/kg tissue water)	Sodium + potassium (m-equiv/kg tissue water)
Unincubated (40)	4·79 ± 0·14	329 ± 10·5	69 ± 2·7	371 ± 20·9	76 ± 4·3	145 ± 5·3
1 hr incubation (30)	4·81 ± 0·03	424 ± 19·8	89 ± 4·0	323 ± 27·5	67 ± 4·9	156 ± 7·6
2 hr incubation (30)	5·38 ± 0·15	565 ± 17·1	106 ± 2·5	326 ± 9·5	61 ± 1·7	167 ± 3·3

The results are given as the mean values ± S.E. of the number of determinations given in brackets.

TABLE 2. The effects of 2 mM cyanide or 2 mM monoiodoacetate on the composition of the rat uterus incubated for 3 hr in Krebs's solution at 37° C

Change in weight (%)	Water (kg/kg dry wt.)	Sodium (m-equiv/kg dry wt.)	Sodium (m-equiv/kg tissue water)	Potassium (m-equiv/kg dry wt.)	Potassium (m-equiv/kg tissue water)	Sodium + potassium (m-equiv/kg tissue water)
Normal −6·4	5·46	526	96	292	53	149
1 mM cyanide +3·1	5·47	502	92	251	45	137
Difference from normal +9·5 ± 1·0†	+0·01 ± 0·22	−24 ± 66·6	−4 ± 1·0	−41 ± 22·0	−8 ± 14·1	—
2 mM iodoacetate +17·3	6·08	756	129	97	15	144
Difference from normal +23·7 ± 1·6‡	+0·62 ± 0·3	+230 ± 59·7‡	+33 ± 6·7†	−195 ± 42·0†	−38 ± 4·6†	—

The values are given as the mean values and mean differences from normal ± S.E. of 10 determinations. Significance of mean differences: † $P < 0{\cdot}01$, ‡ $P < 0{\cdot}001$.

TABLE 3. The effects of low extracellular potassium (1·34 mM) on the composition of the rat uterus incubated for 1 hr in Krebs's solution at 37° C

Change in weight (%)	Water (kg/kg dry wt.)	Sodium (m-equiv/kg dry wt.)	Sodium (m-equiv/kg tissue water)	Potassium (m-equiv/kg dry wt.)	Potassium (m-equiv/kg tissue water)	Sodium + potassium (m-equiv/kg tissue water)
Normal −1·9	4·80	440	92	358	75	167
Low +0·4 potassium	5·03	525	105	297	59	164
Difference from normal +2·3 ± 1·4	+0·23 ± 0·16	+85 ± 25·3†	+13 ± 3·6†	−61 ± 20·3*	−16 ± 1·0‡	—

The values are given as the mean and mean differences ± S.E. of 10 determinations when normal and low potassium treatments were compared using two pieces of uterus from the same rat. Significance of mean differences: * $P < 0{\cdot}05$, † $P < 0{\cdot}01$, ‡ $P < 0{\cdot}001$.

loss of potassium which, if osmotic equilibrium is maintained, could account for the observed increase in weight of the tissue.

Tonicity changes. The sodium chloride concentration in the Krebs's solution was decreased so that the total concentration was 175 m-osmole/l., or increased to give concentrations of 555 or 809 m-osmole/l., the normal concentration in the Krebs's solution being 300 m-osmole/l. The changes in weight of the pieces of uterus during a 3 hr incubation period in these solutions are given in Fig. 2. It can be seen that the tissues all lost weight

TABLE 4. The effects of incubation at 37° C of the rat uterus for 1 hr in modified Krebs's solution containing no calcium or with strontium or magnesium substituted for calcium

	Water (kg/kg dry wt.)	Sodium (m-equiv/kg dry wt.)	Sodium (m-equiv/kg tissue water)	Potassium (m-equiv/kg dry wt.)	Potassium (m-equiv/kg tissue water)	Sodium + potassium (m-equiv/kg tissue water)
Normal (30)	4·81 ± 0·03	424 ± 19·8	89 ± 4·0	323 ± 27·5	67 ± 4·9	156 ± 7·6
No Ca^{2+} (22)	5·91 ± 0·16	142 ± 13·7	24 ± 2·0	182 ± 16·0	31 ± 2·6	55 ± 4·3
Substitution of:						
Sr^{2+} for Ca^{2+} (19)	5·25 ± 0·13	551 ± 21·6	108 ± 5·5	307 ± 18·9	59 ± 3·6	167 ± 4·0
Mg^{2+} for Ca^{2+} (16)	5·48 ± 0·08	566 ± 21·4	105 ± 3·9	315 ± 8·8	58 ± 1·6	163 ± 3·7

The results are given as the mean values ± S.E. of the number of determinations given in brackets.

and that the relative weight decreases were in the order expected on a simple osmotic basis. Pieces of the tissue were analysed for water, sodium and potassium after 1 hr incubation. Although this is not long enough for complete equilibration in (Na + K) content between the tissue and the media, it can be seen that at an external concentration of 175 m-osmole/l. a loss of sodium and potassium from the tissue had occurred. At 555 m-osmole/l. there was an increase in the sodium content of the tissue and a loss of potassium, whereas at 809 m-osmole/l. there was an increase in sodium and also apparently an increase in potassium. It is not possible from the present data to decide whether this increase in potassium is real or whether a loss of tissue solids and water is causing an increase on both a dry-weight and a tissue-water basis. Sections of the tissues confirmed the changes in sizes of the cells indicated by the weight changes and this was clearest in the myometrium.

Cardiac glycosides. Pieces of uterus were incubated for 2 hr in Krebs's solution containing either digoxin (10^{-6} g/ml.) or ouabain (10^{-6} g/ml.). Neither sodium, potassium nor water contents were significantly different from those observed in control experiments where pieces of uterus from the same rat were incubated in normal Krebs's solution for 2 hr (17 experiments in each group).

Oxytocin and histamine. Incubation of pieces of uterus for 2 hr in Krebs's solution containing 3·3 m-u./ml. of oxytocin resulted in a lower potassium

content in these pieces of uterus than in the normally incubated pieces from the same animal. The difference ($46 \pm 12{\cdot}8$ m-equiv/kg tissue water; 22 experiments) was statistically significant ($P < 0{\cdot}01$). There was no significant change in sodium content. Histamine (10^{-6} g/ml.) had no effect in 17 experiments.

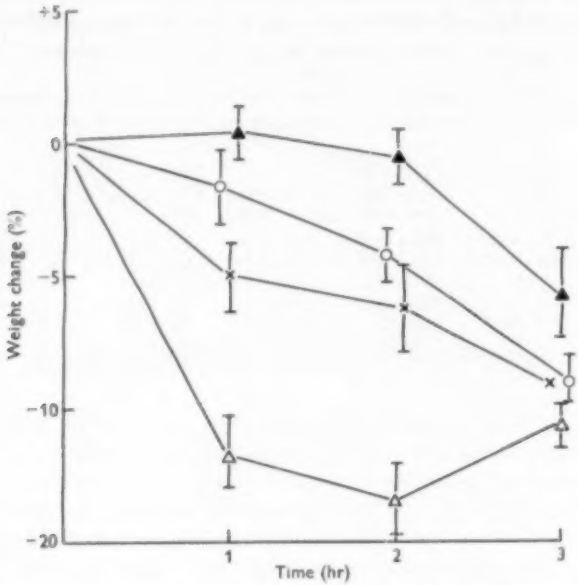

Fig. 2. Changes in weight of pieces of rat uterus incubated in Krebs's solutions of different tonicities (m-osmole/l.); ▲ 175, ○ 300, × 550, △ 809. Each point on the graph represents the mean value of eight experiments and the vertical lines indicate the standard error. Note change in ordinate scale.

TABLE 5. The effects of changes in tonicity of Krebs's solution on the composition of the rat uterus after 1 hr incubation at 37° C

Treatment	Water (kg/kg dry wt.)	Sodium (m-equiv/kg dry wt.)	Sodium (m-equiv/kg tissue water)	Potassium (m-equiv/kg dry wt.)	Potassium (m-equiv/kg tissue water)	Sodium + potassium (m-equiv/kg tissue water)
Hypotonic, 175 m-osmole/l. (10)	$4{\cdot}80 \pm 0{\cdot}123$	$270 \pm 36{\cdot}7$	$57 \pm 3{\cdot}5$	$169 \pm 24{\cdot}6$	$36 \pm 5{\cdot}6$	$93 \pm 8{\cdot}1$
Hypertonic, 555 m-osmole/l. (6)	$4{\cdot}07 \pm 0{\cdot}254$	$705 \pm 32{\cdot}0$	$174 \pm 4{\cdot}0$	$182 \pm 20{\cdot}0$	$46 \pm 5{\cdot}8$	$220 \pm 7{\cdot}8$
809 m-osmole/l. (10)	$3{\cdot}98 \pm 0{\cdot}112$	$1029 \pm 34{\cdot}6$	$261 \pm 15{\cdot}3$	$403 \pm 23{\cdot}8$	$102 \pm 6{\cdot}5$	$363 \pm 21{\cdot}0$

For normal values (300 m-osmole/l.) see Table 1. Results are given as the mean values ± S.E. of the number of determinations given in brackets.

DISCUSSION

Tissues such as liver and kidney swell when incubated in artificial media consisting mainly of sodium chloride (see Robinson, 1960). The oestrous rat uterus, however, lost weight when incubated in normal Krebs's solution. As this was not associated with a reduction in water content expressed on a dry-weight basis, it must be due to a loss of tissue solid and accompanying water. When swelling is observed in isolated tissues incubated in various solutions it is generally attributed to a greater uptake of sodium and chloride than loss of potassium. In the rat uterus a gain of sodium in excess of the loss in potassium is in fact observed, so that in this respect the tissue is behaving in a way essentially similar to that of liver and kidney. Both cyanide and iodoacetate when added to the bathing Krebs's solution cause a swelling of the tissue, but only with iodoacetate can this be correlated with a net gain of sodium. The failure of cyanide to affect the movement of sodium and potassium may have been due to the inadequacies of the experiment, but it is possible that sodium and potassium exchanges are energized by glycolysis as in frog striated muscle (Frazier & Keynes, 1959). The effects of low potassium concentration also indicate that there is a coupled sodium–potassium exchange as in striated muscle, nerve and erythrocytes (see Ussing, 1960). The lack of action of cyanide, and the effect of a low external potassium concentration on the sodium content of the rat uterus, are consistent with the observations of Daniel & Robinson (1960*a*, *b*) on sodium extrusion from the rabbit and cat uterus.

In the absence of calcium from the bathing solution the tissue took up water. In addition there was a loss of both sodium and potassium so that the (Na + K) content/kg tissue water was much lower than that in the bathing solution. The extracellular space was not determined but photomicrographs showed an increase in cell size and if this was accompanied by a decrease in extracellular space it could at least partly account for the loss of sodium that was observed. Bozler & Lavine (1958), using frog stomach muscle, have also described a net loss of both sodium and potassium in the absence of calcium. If we assume that water movement in tissues is principally the result of differences in osmotic pressure (Robinson, 1960) then the swelling in the absence of calcium would seem to be due to an increase in the amount of non-diffusable molecules (idiomolar value, Conway & Hingerty, 1946) in the cell. Strontium and magnesium substituted for calcium maintain the (Na + K) content at a level similar to that of the bathing solution. They do not, however, prevent the swelling which in this case seems due to a gain of sodium. Calcium may thus have at least two actions in the rat uterus: the maintenance of (1) the idiomolar value and (2) the integrity of the cell membrane and of the 'sodium pump'.

With regard to the first effect the rat uterus is known to have a larger and more labile idiomolar value than skeletal muscle (Cole, 1950, 1951) and this is affected by oestradiol (Cole, 1950). Calcium is known to have a 'stabilizing' effect on proteins (Gurd & Wilcox, 1956), and as calcium is bound to intracellular structures forming bridges between anionic groups (Haurowitz, 1956) a loss of calcium may result in a break-down of organic molecules and increased idiomolar value. Calcium is also known to be linked with sodium transport in certain erythrocytes (Maizels, 1954), toad urinary bladder (Bentley, 1960) and the rat ileum (Dumont, Curran & Solomon, 1960). Daniel & Robinson (1960*a*) on the other hand, found that absence of calcium from the bathing media did not affect the rate of sodium extrusion from rabbit or cat uterus.

Cardiac glycosides inhibit the active extrusion of sodium from erythrocytes, giant nerve axons, striated muscle and mouse ascites tumour cells (see Ussing, 1960). They have also variable effects on the passive movements of sodium and potassium. In the rat uterus neither digoxin nor ouabain had any effect on the sodium or potassium content. The possibility cannot be ruled out, however, that the influx and efflux of these ions were inhibited to a similar degree, as was demonstrated in mouse ascites tumour cells by Maizels, Remington & Truscoe (1958).

Oxytocin depolarizes and contracts uterine muscle (Thiersch, Landa & West, 1959). Born & Bulbring (1956) have shown that there is an increase in the rate of efflux of potassium from guinea-pig taenia coli when it was stimulated with acetylcholine or histamine. There was a net loss of potassium from the rat uterus when it was in contact with oxytocin but no change in the sodium content could be demonstrated. Histamine on the other hand had no effect on sodium or potassium and it also has no effects on the contractility of the rat uterus.

SUMMARY

1. The oestrous rat uterus gains sodium and loses potassium when incubated in Krebs's solution. This process is accelerated by 2 mM iodoacetate and a low extracellular potassium concentration. Cyanide had no statistically significant effect.

2. When calcium was excluded from the Krebs's solution the tissue swelled and there was a decrease in the amounts and total (Na + K)/kg tissue water in the tissue. Strontium or magnesium when substituted for calcium reduced the swelling and abolished the net loss of total electrolytes (sodium + potassium). Sodium nevertheless accumulated at an increased rate compared to tissue incubated in normal Krebs's solution.

3. Neither digoxin nor ouabain had any effect on the amounts of sodium or potassium in pieces of uterus.

4. Oxytocin decreased the potassium content of the uterine tissue but had no detectable effect on the amount of sodium present.

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STUDIES OF LEPTOMENINGEAL COLLATERAL CIRCULATION IN *MACACUS RHESUS*

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In previous experiments on dogs (Symon, 1960), it was shown that, within a few minutes of occlusion of a major cerebral vessel, free flow of blood occurred within the area of supply of the occluded vessel, borne via leptomeningeal anastomoses from the vascular fields of adjacent vessels. Since it has been established anatomically (Vander Eecken, 1959) that the number and size of collateral vessels within the pial circulation decreases with increasing convolution of the cortex in higher animals, it became of interest to find out whether such prompt collateral flow could be demonstrated in these animals as well. Similar experiments were therefore carried out in the primate *Macacus rhesus*.

METHODS

Eighteen macaque monkeys weighing between 3 and 5 kg were used. They were anaesthetized with intravenous thiopentone sodium in an initial dose of 25 mg/kg, supplemented at intervals by intravenous doses of 25 mg as required. An endotracheal tube was passed to facilitate maintenance of a clear airway throughout. The blood pressure was continuously recorded from the cannulated femoral artery.

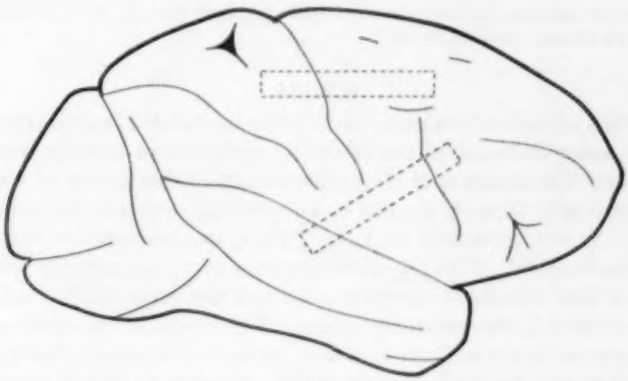
Dissection of the carotid sinus. The area of the right carotid sinus was exposed, and the carotid-sinus nerve divided. No ligations of vessels were necessary, since in the macaque, unlike the dog, the main route for blood from the area of the carotid sinus to the cerebral cortex is via the internal carotid artery, and anastomoses between intra- and extra-cranial vessels are small and functionally unimportant.

A polythene cannula with dead space 0·1 ml. was introduced into the lingual artery and threaded for a few millimetres down the external carotid artery, so that its tip lay in the carotid sinus, care being taken not to occlude the origin of the internal carotid artery. Injections of 0·5 ml. of labelled erythrocyte suspension were made through this cannula from a tuberculin syringe. These injections were made as rapidly as possible. After each injection the cannula was flushed out and filled with a solution of heparin (500 u./ml.). In some experiments this dissection was performed on both sides.

Exposure and occlusion of the vertebral arteries in the neck. A curved transverse incision was made half an inch (12 mm) above the sternoclavicular joints, extending for 2·5 cm on either side of the mid line. Both sternomastoid muscles were divided and the carotid sheaths gently retracted medially. The origin of scalenus anterior was defined, behind the carotid sheath, arising from the transverse processes of the cervical vertebrae. The vertebral artery was found in the interval between the longus cervicis muscle on the vertebral bodies and

the scalenus anterior passing downwards and laterally, as it ascended to enter the transverse process of the sixth cervical vertebra. The vessel could then be clipped or tied close to its origin from the subclavian artery.

Cannulation of the vertebral artery. The axilla was opened by an incision extending through skin and muscle from the sternoclavicular joint downwards and laterally to the anterior border of the axilla. After clearing the muscles from both borders of the medial end of the clavicle and dividing the external jugular vein, the sternoclavicular joint was dislocated and the clavicle retracted laterally with division of the lateral head of sternomastoid in the process. The axillary artery was cleared upwards from the axilla, and followed to the arch of the subclavian artery, where the origin and first part of the vertebral artery could be defined. A catheter, of dead space 0·1 ml., was then passed up from the axillary artery into the subclavian, and manipulated into the origin of the vertebral artery.



Text-fig. 1. Lateral view of the right cerebral hemisphere of *Macacus rhesus*. The dotted rectangles indicate the positions in which the Geiger counter was placed.

Insertion of the Geiger counter. The animal was turned onto its left side and a U-shaped skin flap was made, based posteriorly and extending from the external auditory meatus forwards along the zygoma, along the orbital margin to the mid line and backwards to just medial to the pinna. The temporal muscle was excised and a wide craniectomy made from the mid line to the temporal fossa. The dura was opened by a T-shaped incision and the dural arteries were coagulated with diathermy. Occasionally a large vein emerging from the veins of the Sylvian fissure, and passing directly into the dura, was found during the reflexion of the dura, and was clipped. For recording the flow of labelled erythrocytes through the cerebral cortex a probe-pattern Geiger counter (20th Century Electronics Type N 1b) of 3 mm external diameter was introduced obliquely in one of the two positions shown in Text-fig. 1, so that the depth of the centre of the counter tip was some 5 mm from the pial surface. In most experiments one counter position only was used throughout. When both were used, the first insertion was in the lateral of the two sites. The output of the probe passed to a small cathode follower, and thence to a rate-meter and Evershed pen recorder. A paper speed of 12 in. (30 cm)/min was used.

Dissection of the basal vessels for occlusion of the anterior and middle cerebral vessels. Gentle retraction of the temporal pole backwards enabled the Sylvian fissure to be opened, and the arachnoid membrane overlying the optic nerve to be breached by the tip of a fine sucker. This revealed the terminal 5–6 mm of the internal carotid artery, with the almost right-angled division into middle and anterior cerebral arteries lying lateral to the optic nerve.

Interruption of blood flow in the anterior and middle cerebral arteries was carried out as described previously (Symon, 1960).

Red-cell labelling. Red cells were labelled by the method described by Mollison, Robinson & Hunter (1958) and suspended in 0·9 % NaCl solution to give a final activity of 10 μc/ml. Immediately before use 0·1–0·2 ml. of this solution was diluted to 0·5 ml. with 0·9 % NaCl solution. When the labelled red cells were injected into the lingual artery an initial injection was made to assess the sensitivity. An appropriate activity was then selected and each subsequent lingual injection was of the same activity and volume. When injections were made into the vertebral artery, the activity and volume of injection was doubled.

Perfusion technique. In order to confirm details of occlusion of the vessels, and to obtain information about the presence or absence of abnormality of the circle of Willis and areas to which the vascular supply had been reduced, the head was perfused at the end of each experiment with carmine gelatine, according to the method described for the dog (Symon, 1960). In the macaque, smaller volumes of perfusion fluid (600 ml., 3·8 % sodium citrate and carmine gelatine) were employed.

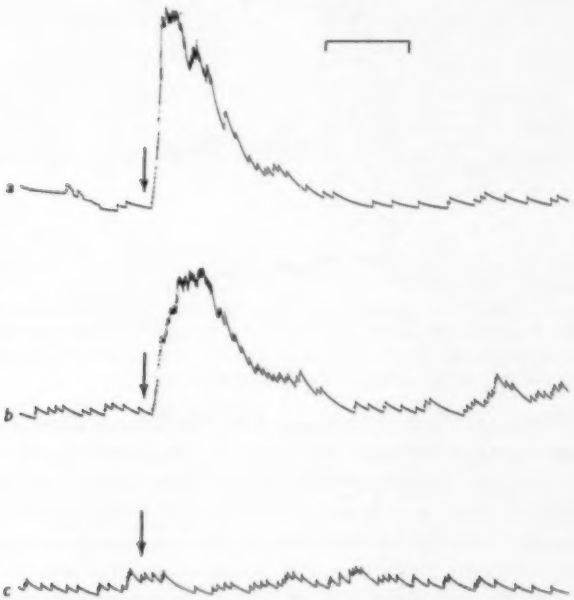
RESULTS

Injection of radioactive material into the cannulated lingual artery produced a sharp deflexion of the recording pen with an average latency of 0·4–0·6 sec. The duration of the deflexion reflects the period of transit of radioactive cells through the pial vessels and the cortex in the area of the counter. It was sustained for 6·25–8·75 sec, as compared to 9–13 sec in dogs (Symon, 1960). With repeated injections of a given activity, provided the speed and volume of injection remained the same, similar deflexions were produced in the recording system. The height of the deflexion was closely related to the activity injected. More accurate reproducibility was obtained in the macaque than had been possible in the dog, probably because the route of transit of the active material from cannula to cortex was directly via the internal carotid artery, whereas in the dog it was less direct, via the arteria anastomotica.

Occlusion of anterior and middle cerebral arteries

With the counter in either of the positions shown in Text-fig. 1, occlusion of the anterior cerebral artery produced little change in the form of the deflexion. Such an experiment is illustrated in Text-fig. 2. In *a* the deflexion is shown with the circulation intact, and in *b* after occlusion of the anterior cerebral artery by silver clip between 1 and 2 mm from its origin from the internal carotid artery. The latency of the deflexion remains, within the limits of the experiment, the same, although the deflexion rises to peak height more slowly, and is slightly prolonged. Similar slight changes are obtained, without occlusion of the anterior cerebral artery or other vascular interference, by repeated or forceful retraction of the brain or exposure of the basal vessels and freeing from the surrounding arachnoid. They probably represent minor alterations in the blood flow in pial vessels and cortex, not associated with any specific vascular occlusion.

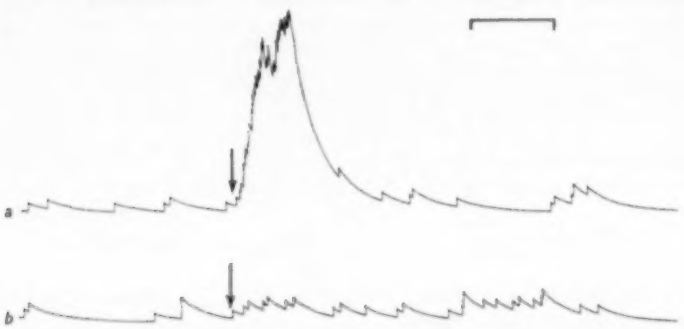
Record *c* was made after the middle cerebral artery had also been occluded by clip close to its origin. There was no residual carotid blood flow through the observed cortical area. Essentially the same result had been obtained in the dog; therefore in both species this area of cortex draws its normal carotid blood supply from the middle cerebral artery.



Text-fig. 2. Records of radioactivity from the right frontoparietal cortex of a macaque. At each arrow, injection of ^{32}P-labelled red cells was made into the lingual cannula: at *a* with the cerebral circulation intact, at *b* after occlusion of the right anterior cerebral artery, and at *c* after occlusion of right middle cerebral artery as well. Time marker in *a*, 5 sec.

After occlusion of the middle cerebral artery the residual flow detected was found to vary with the conditions of the experiment. In seven animals in which the middle cerebral artery was occluded within 2 mm of its origin by a silver clip, the ipsilateral carotid artery was the only vessel exposed in the neck. In these animals little evidence of residual flow of ipsilateral carotid origin was detected in the area of cortex studied following an injection of radioactive cells into the ipsilateral carotid circulation within 5 min of middle cerebral occlusion. An experiment to illustrate this result is shown in Text-fig. 3. At *a* is shown the control deflexion with intracranial vessels intact, and at *b* the result following the injection of activity 4 min

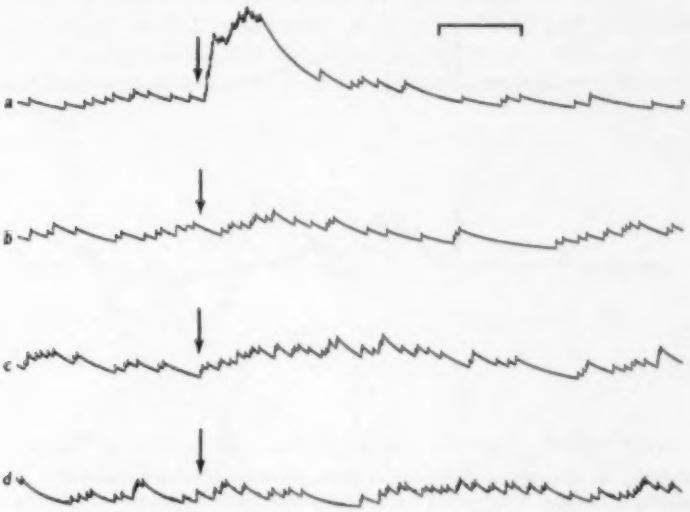
after occlusion of the middle cerebral artery. There is no longer a deflexion, although the injection is followed by a slight but perceptible increase in rate of count, suggesting that the ipsilateral carotid flow has not completely disappeared.



Text-fig. 3. Records of radioactivity from the right frontoparietal cortex of a macaque. At each arrow, injection of ^{32}P-labelled red cells was made into the lingual cannula: at *a* with the cerebral circulation intact, and at *b* 4 min after occlusion of the right middle cerebral artery. Time marker in *a*, 5 sec.

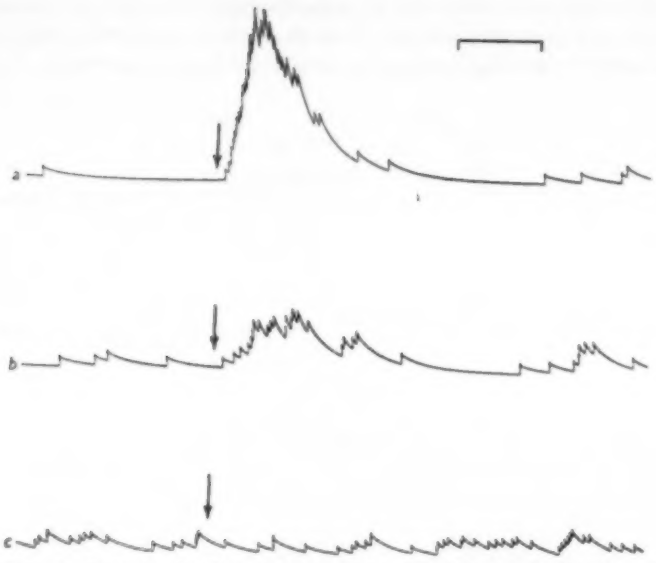
In four of these animals it was possible to follow the collateral circulation after middle cerebral occlusion for about 30 min, since the blood pressure remained constant throughout this period. The cortex and pial vessels were protected from drying and clotting with lintine strips soaked in warmed Locke's solution. In three of these experiments, there was evidence that some collateral flow from the anterior cerebral artery developed following middle cerebral occlusion. In the fourth there was no such evidence, although the cortex and pial circulation were apparently in good condition. The result of one of the three experiments in which some collateral flow developed is illustrated in Text-fig. 4. The effects of lingual injection are shown at *a*, with the cerebral circulation intact, and at *b* and *c*, 10 and 30 min after application of the middle cerebral clip. The slight increase in counting rate following injection in *b* is enhanced in *c*. Comparing the count rate in the 10 sec period immediately following injection with that of a 10 sec background averaged over the previous half minute, there is an increase in counts over background of 64 % in *b*, and 130 % in *c*. With the anterior cerebral artery also occluded, such a rise in the rate of count after injection is no longer seen (*d*). The period of observation was not extended for longer than 30 min because of the difficulty of keeping the preparation in good condition. It is therefore not possible to state whether full development of ipsilateral anterior cerebral collateral circulation had been reached. The experiments suggest, however, that in the half hour

following middle cerebral occlusion some change in the cerebral circulation occurred, so that a collateral flow from the anterior cerebral artery, which could hardly be detected soon after occlusion, became manifest.



Text-fig. 4. Records of radioactivity from the right frontoparietal cortex of a macaque. At each arrow, injection of ^{32}P-labelled red cells was made into the lingual cannula: at *a* with the cerebral circulation intact, at *b* and *c* 10 and 30 min respectively after occlusion of the right middle cerebral artery, and at *d* after occlusion of the right anterior cerebral artery as well. Time marker in *a*, 5 sec.

When occlusion of the middle cerebral artery was carried out after ligation of both vertebral arteries, there was a much greater residual flow of ipsilateral carotid origin. In two experiments, the result of one of which is shown in Text-fig. 5, both vertebral arteries were ligated low in the neck close to their origin from the subclavian arteries, 2 hr before the dissection of the intracranial blood vessels. This procedure did not affect either blood pressure or respiration. In *a* is shown the effect of lingual injection before occlusion of the middle cerebral artery. The deflexion does not differ from that seen in corresponding experiments with the vertebral arteries intact. In *b* is shown the effect of lingual injection 4 min after the middle cerebral artery had been clipped. The deflexion has an increased latency, a more gradual rise, a great reduction of peak height, and a slight prolongation. As is seen by comparison with Fig. 4, this residual flow is greater than when the vertebral arteries are intact. It must originate from the ipsilateral anterior cerebral artery, since after occlusion of this vessel, the lingual injection no longer produces a deflexion (*c*).



Text-fig. 5. Records of radioactivity from the right frontoparietal cortex of a macaque, in which both vertebral arteries had been ligated 2 hr previously. At each arrow, injection of ^{32}P-labelled red cells was made into the lingual cannula: at *a* with the intracranial circulation intact, at *b* 4 min after occlusion of the right middle cerebral artery, and at *c* after occlusion of the right anterior cerebral artery also. Time marker in *a*, 5 sec.

There are two possible explanations of this increase in collateral flow:

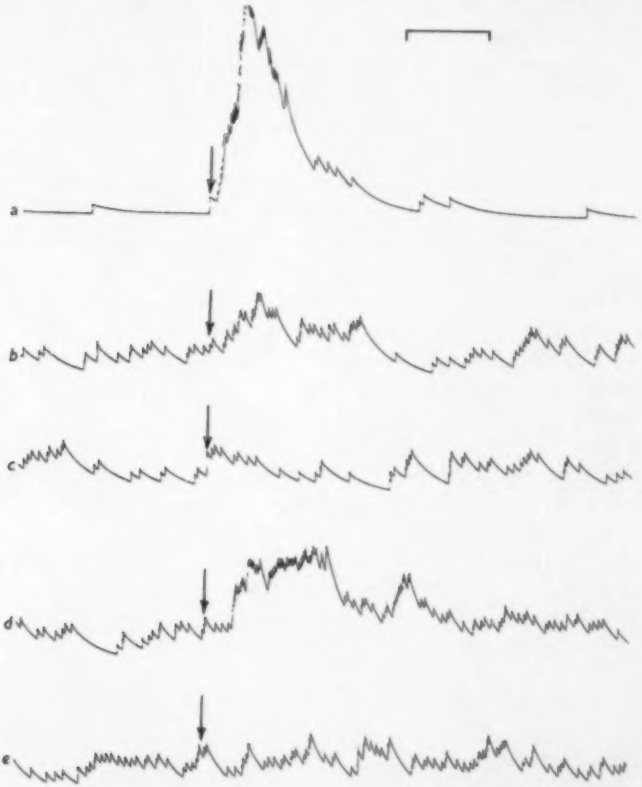
First, with the decrease in flow from the posterior cerebral artery consequent upon occlusion of the vertebral arteries a greater part of the cortical field of the middle cerebral artery is supplied by the anterior cerebral artery after the middle cerebral artery has been occluded.

Secondly, it is known that after occlusion of a main vessel in the neck, dilation of other vessels to the brain may occur (MacDonald & Potter, 1950; Stenik, 1954). If this were to happen after occlusion of the vertebral arteries, a greater collateral flow from the anterior cerebral artery could be expected to occur into the field of the middle cerebral artery when it was subsequently occluded.

Evidence of cross-flow from the contralateral carotid circulation. A special feature of the cerebral circulation in the macaque is the junction of the two anterior cerebral arteries in a common pericallosal artery. In order to assess any possible cross-flow from the contralateral anterior cerebral artery via the common pericallosal artery and leptomeningeal anastomosis

into the field of the occluded middle cerebral artery, two animals were prepared with bilateral carotid dissection and cannulation of both lingual arteries. After the middle cerebral artery had been occluded, labelled red cells could be injected into the opposite lingual artery.

The result of one such experiment is shown in Text-fig. 6. The effect of injection of active cells into the right lingual artery with the middle cerebral artery intact is seen at *a*, and 4 min after occlusion of the middle



Text-fig. 6. Records of radioactivity from the right frontoparietal cortex of a macaque, in which both carotid sinus nerves were divided and both lingual arteries cannulated. At the arrows in *a*, *b* and *c*, injection of ^{32}P-labelled red cells was made into the right lingual cannula: in *a* with the cerebral circulation intact, in *b* 4 min after occlusion of the right middle cerebral artery, and in *c* after occlusion of the right anterior cerebral artery also. At the arrows in *d* and *e*, injection of ^{32}P-labelled red cells was made into the left lingual cannula: in *d* after right middle and anterior cerebral occlusion, and in *e* after division of leptomeningeal anastomotic vessels on the surface of the right hemisphere also. Time marker in *a*, 5 sec.

cerebral artery at *b*. Evidence of a definite collateral flow is shown by a delayed deflexion of lowered peak height. This collateral flow is greater than in the corresponding experiments in which only the ipsilateral carotid artery had been dissected in the neck. The difference may be accounted for by the fact that in these two preparations the arterial blood pressure is higher, owing to the section of both carotid-sinus nerves. Another possible factor is the extensive manipulation of the carotid artery involved in division of the carotid-sinus nerve, which tends to cause vascular spasm in the area of the carotid sinus. If this occurs only on the side of subsequent middle cerebral occlusion, the greater flow from the opposite carotid artery would obscure the collateral flow from the isilateral anterior cerebral artery to a greater extent than if such spasm were to affect both carotid arteries.

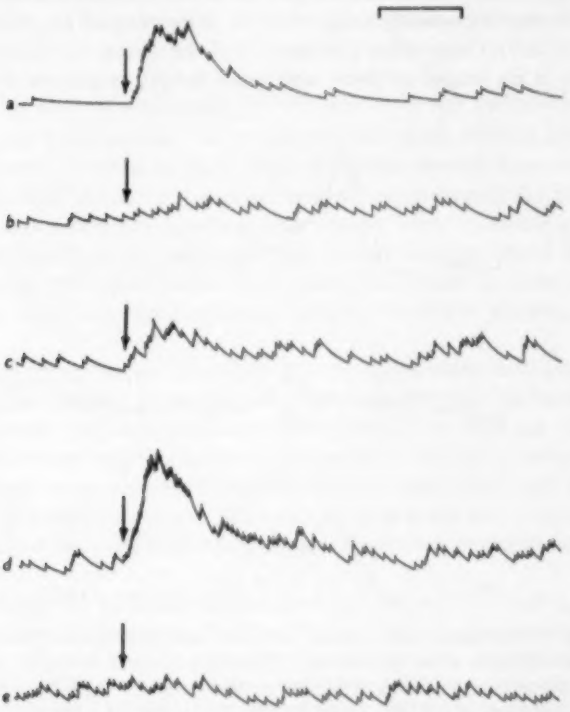
As shown in Text-fig. 6*c* this residual flow must have its origin from the ipsilateral anterior cerebral artery, since it is abolished by occlusion of this vessel. An injection into the contralateral lingual cannula still produces a clear deflexion as shown in *d*. The deflexion of contralateral origin has about the same latency as that recorded after ipsilateral injection in *b*, but its peak height is appreciably greater. This suggests that following middle cerebral occlusion by clip on the right side some degree of vascular spasm has occurred in the right anterior cerebral artery, while the left anterior cerebral artery, which has not been exposed, remains unaffected.

The residual collateral flow shown in *d* arises from the contralateral anterior cerebral artery, since it largely disappears when leptomeningeal anastomotic vessels between the pericallosal artery and the occluded middle cerebral artery are divided by a parasagittal incision into the cortex medial to the area of insertion of the counter, from the frontal pole backwards to the post-parietal region. Thereafter an injection of active cells into the contralateral lingual cannula no longer produces an obvious deflexion, as shown in *e*.

Evidence of spasm in the basal vessels of the macaque

Spasm in basal vessels in the macaque on mechanical stimulation has been reported by Harvey & Rasmussen (1951). In the experiments of the present series spasm was often observed with the naked eye during exposure and occlusion of the middle cerebral artery. This spasm involved the terminal part of the internal carotid artery and the first 4–5 mm of the course of the anterior and middle cerebral arteries. There was no apparent change in the vessels on the surface of the hemisphere. Even when no narrowing of the basal vessels could be seen with the naked eye, alterations in the pattern of flow in the cortex following lingual injection could be observed, suggesting the presence of some spasm.

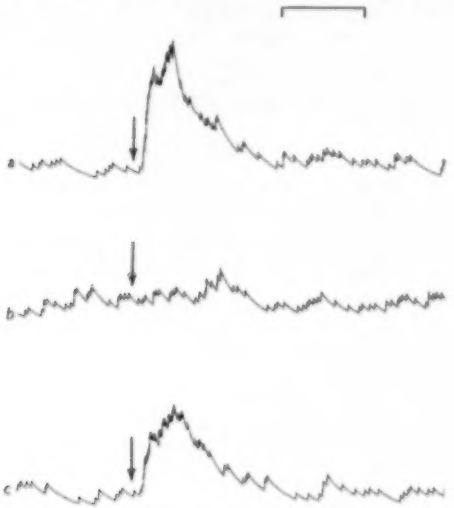
Different degrees of this spasm were demonstrated in four animals as follows: A light spring clamp was applied for 5 min to the first few millimetres of the middle cerebral artery, after the vessel had been freed from the overlying arachnoid. On removal of the clip the affected segment of



Text-fig. 7. Records of radioactivity from the right frontoparietal cortex of a macaque. At each arrow injection of ^{32}P-labelled red cells was made into the lingual cannula: at *a* with the cerebral circulation intact, at *b* after application of a light spring clamp to the right middle cerebral artery, at *c* and *d* 10 and 30 min after removal of the clamp, and at *e* after occlusion of the right middle cerebral artery by silver clip. Time marker in *a*, 5 sec.

the middle cerebral artery showed considerable 'spasm' in two, and lesser degrees of narrowing in the other two experiments. The result of one of the experiments in which appreciable narrowing occurred is shown in Text-fig. 7. The trace in *a* shows the effect of the control injection, after the middle cerebral artery had been freed from the arachnoid in the vicinity of its origin, and in *b*, with the light spring clamp applied to the vessel close to its origin from the internal carotid artery. There is little evidence

of flow of ipsilateral carotid origin on injection of active cells. In *c* is shown the activity on injection 10 min after the light clamp was removed. There is delay in arrival of activity and the peak height of the deflexion is decreased. This indicates a reduction in carotid flow through the area studied. Appreciable reduction in calibre of the middle cerebral artery in the area of the clip was still apparent on inspection of the vessel at this time. In *d*, half an hour after the removal of the clamp, the delay in arrival of activity is no longer evident, and peak height is greater than in the



Text-fig. 8. Records of radioactivity from the right frontoparietal cortex of a macaque. At each arrow injection of ^{32}P-labelled red cells was made into the lingual cannula: at *a* with the cerebral circulation intact, at *b* after application of a light spring clamp to the right middle cerebral artery, and at *c* 4 min after removal of the clamp. Time marker in *a*, 5 sec.

control trace *a*. By this time no trace of narrowing in the area of the temporary clip was apparent. The significant increase in trace height in *c* as compared to the control may indicate that mobilization of the vessel alone, before the control record was taken had produced some degree of spasm in the middle cerebral artery, which had disappeared when record *d* was taken. Record *e* was taken 4 min after permanent occlusion of the middle cerebral artery by clip. All trace of flow has disappeared.

Text-figure 8 is from one of the two experiments in which temporary occlusion of the middle cerebral artery had produced little visible spasm. In *a* is shown the control trace after mobilization of the vessel. Trace *b* was obtained with the spring clip in place; there is little evidence of collateral

flow of ipsilateral carotid origin. In *c*, some 4 min after removal of the clip, there is no delay in arrival of activity but some reduction in peak height. On inspection of the artery no definite narrowing could be seen at the site of the clip.

In all experiments where spasm was noticed, it wore off, as evidenced by flow recording or inspection or both, in from 20–40 min, according to the degree.

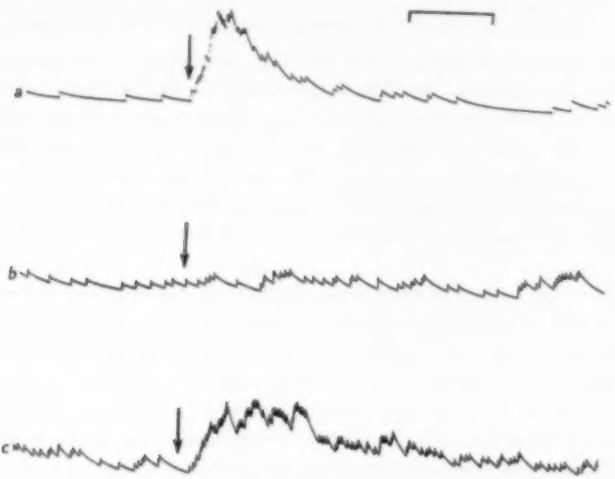
Injection of active cells into the vertebrobasilar circulation

In the macaque monkey, as in the dog, occlusion of both anterior and middle cerebral arteries at their origin from the carotid does not result in cessation of flow in the small pial arteries on the surface of the hemisphere, since in this condition bleeding still occurs when a small pial artery is cut. A possible entry route for this slowed residual blood flow, other than from the proximal part of the contralateral anterior cerebral artery, is via the leptomeningeal collateral vessels from the posterior cerebral artery.

Evidence for collateral flow from the vertebrobasilar circulation is provided by the results of the following two experiments. After the middle cerebral artery had been occluded, and carotid flow through the studied area of the cortex largely abolished, the origin of the right vertebral artery was exposed by a combined cervico-axillary dissection, and a catheter threaded up from the axillary artery to enter and become impacted in the origin of the right vertebral artery, so as to inject active cells directly into this vessel.

The records of such an experiment are shown in Text-fig. 9. The deflexion at *a* was produced by injection into the lingual cannula. In *b* is shown the effect of lingual injection after the middle cerebral artery had been occluded some 30 min previously. Little carotid flow is occurring through the area under study. The deflexion in *c* was produced by injection directly into the right vertebral artery 35 min after occlusion of the middle cerebral artery. The activity was twice that injected into the lingual cannula, and the injected volume was also doubled. This was found necessary with vertebral injections in order to produce an adequately sharp and demonstrable peak deflexion, because of the increased dilution and dispersal to which the injected activity is subject in its greater length of travel between the thoracic inlet and the cortex. The latency of the deflexion in *c* appears to be two or three times as great as that following the control lingual injection; the peak of the deflexion is delayed, the peak height is less than that produced by lingual injection of half the activity, and the whole deflexion, allowing for the increased volume injected, is appreciably prolonged.

This experiment indicates that appreciable collateral flow may occur in the lower part of the middle cerebral field from the vertebrobasilar circulation even when there is little evidence of collateral blood flow of carotid origin. The only possible route for this collateral flow is from the posterior cerebral artery via leptomeningeal anastomoses. Such collateral flow becomes increasingly difficult to demonstrate as the counter position is moved higher and more medially in the hemisphere.



Text-fig. 9. Records of radioactivity from the right frontoparietal cortex of a macaque. At the arrows in *a* and *b* injection of ^{32}P-labelled red cells was made into the lingual cannula: at *a* with the cerebral circulation intact, and at *b* 30 min after occlusion of the right middle cerebral artery. At the arrow in *c* twice the activity of ^{32}P-labelled red cells in twice the volume previously used was injected into the right vertebral artery. Time marker in *a*, 5 sec.

Perfusion of the brain

When the brain was perfused after occlusion of both the middle and anterior cerebral arteries by clip or diathermy, the intensity of perfusion of the parietal and temporal lobes, and of the insula was slightly reduced. The findings were similar to those in the dog. In the basal ganglia, however, there were minor differences between the macaque and the dog which reflected the slightly different distribution of vessels within the deep nuclei in the two species.

When the middle cerebral artery was occluded by clip, leaving its perforating branches distal to the clip intact, there was little difference in the intensity of perfusion of the basal ganglia between the operated and the non-operated side. On the other hand, when the middle cerebral artery

was occluded by diathermy, and excised as far as its proximal bifurcation, with occlusion of its central branches, certain areas within the basal ganglia failed to be perfused. As in the dog, the maximal reduction in intensity of perfusion was in the body and caudal part of the head of the caudate nucleus, and in the putamen of the lentiform nucleus, whereas the rostral part of the head of the caudate nucleus was perfused from the perforating branches of the anterior cerebral artery. In contrast to the findings in the dog, however, even after occlusion of the central branches of the middle cerebral artery there was good perfusion of the medial part of the globus pallidus, and of the tail of the caudate nucleus, and incomplete perfusion of the amygdaloid nucleus, structures which in the dog were not perfused under these conditions. The different origins of the anterior choroidal artery in the two species may account for this. In the dog the anterior choroidal artery is a branch of the proximal few millimeters of the middle cerebral artery, and is thus occluded in diathermy excision of this part of the middle cerebral artery, whereas in the macaque, the anterior choroidal artery springs directly from the internal carotid artery close to its termination, so that it is not occluded with the central branches of the middle cerebral artery supplying the major part of the basal ganglia. Therefore the tail of the caudate nucleus, the amygdala and the medial part of the globus pallidus, which are supplied by the anterior choroidal artery, are well perfused. These findings are illustrated in representative sections of a macaque brain in Pl. 1. The brain of the macaque shown in Pl. 1 was perfused after occlusion of the right anterior and middle cerebral arteries by clip at their origin, and diathermy excision of the middle cerebral artery from the clip to just short of its proximal bifurcation, with occlusion of its central branches. In section 1*a* the upper lateral part of the head of the right caudate nucleus is pale, while no significant differences are seen in the rest of the brain between operated and non-operated sides. In section 1*b*, cut more caudally, the rostral part of the lentiform nucleus and the body of the caudate nucleus on the operated side are not perfused. The medial part of the conjoined caudate and lentiform mass, rostral to the lower part of the internal capsule is still perfused. In section 1*c*, more caudally still, the lentiform nucleus, the lateral part of the globus pallidus and the body of the caudate nucleus are not perfused on the operated side. Sections 1*d*, *e* and *f*, are cut progressively more caudally, and show that as the differentiation between globus pallidus and putamen becomes more apparent, the medial part of the right globus pallidus is well perfused. The increasing degree of perfusion of the body and tail of the right caudate nucleus, as one proceeds caudally, is also apparent. The amygdala is incompletely perfused, as is seen in sections 1*d* and *e*. The claustrum takes its blood supply from the middle cerebral artery via the most lateral group

of perforating branches, which arise close to the proximal bifurcation of the middle cerebral itself and have not been occluded in this preparation. It is perfused on the operated side, as is seen in sections 1*d*, *e* and *f*. In the brain of a macaque perfused after occlusion of the middle and anterior cerebral arteries by clip only, no significant differences were apparent in the intensity of perfusion between the two sides.

As in the dog, occlusion of the middle cerebral artery by clip or diathermy produced no differences in the intensity of perfusion of mid-line diencephalic or more caudal structures.

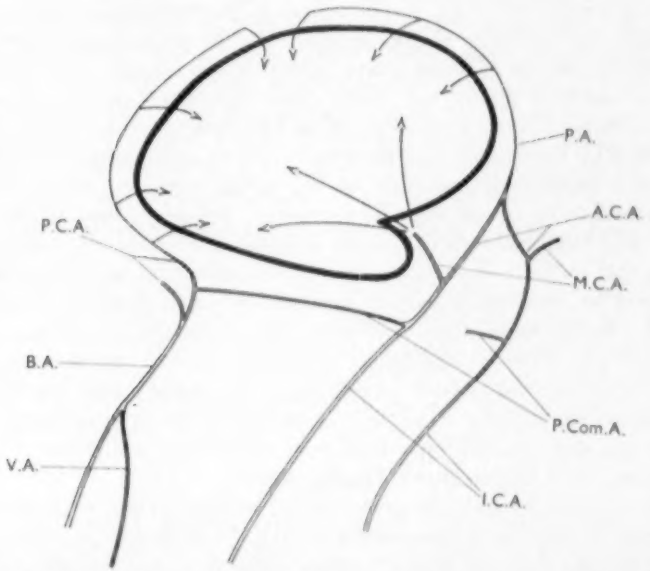
DISCUSSION

The demonstration that, within a few minutes of middle cerebral occlusion in the macaque, blood flow becomes established in the pial circulation within the field of the occluded artery is in agreement with the findings of Petersen & Evans (1937) and Meyer (1958), who demonstrated on microscopic observation a slowed but perceptible flow in these vessels after middle cerebral occlusion. The pattern of collateral circulation which develops in the macaque differs from that in the dog (Symon, 1960). In the latter species within a few minutes of middle cerebral occlusion free blood flow occurs into the field of the occluded vessel from the ipsilateral anterior cerebral artery. In the macaque there is as a rule little evidence of flow from the ipsilateral anterior cerebral artery into the territory of the occluded middle cerebral artery, collateral flow being established from other vessels.

A possible explanation for this species difference might be that there are fewer anastomotic connexions between the anterior and middle cerebral arteries in the macaque than in the dog. Vander Eecken (1959) has pointed out that anastomoses between the two vessels become fewer and smaller with a more highly developed neopallium. However, this explanation alone is not sufficient. It does not account for the appreciable collateral flow from the ipsilateral anterior cerebral artery into the occluded middle cerebral field in the experiments in which the vertebral arteries had been ligated, or in those in which both carotid sinuses had been exposed and the carotid sinus nerves divided so as to make injections into either lingual artery. The explanations which have been put forward when describing the results of these experiments depend on the view that, in the pial circulation, the area of supply of each cerebral vessel may vary with variations in the pressure relationships between the major cerebral arteries feeding into this network. After occlusion of the middle cerebral artery, the area supplied by the anterior and posterior cerebral arteries will expand to cover a proportion of the middle cerebral field, and the proportion taken over by each will depend on the pressure

and volume of flow available within its own field at the moment of occlusion.

In the experiments in which both vertebral arteries were ligated intravascular pressure in the vertebrobasilar circulation was certainly reduced. The fact that the ligation has no effect on blood pressure or respiration shows that flow in the medullary part of the basilar artery must still continue, either by way of the circle of Willis or by anastomoses in the intervertebral foramina between the vertebral arteries and muscular branches



Text-fig. 10. Schematic diagram of the basal arteries of the macaque brain. I.C.A. Internal carotid artery. M.C.A. Middle cerebral artery. A.C.A. Anterior cerebral artery. P.C.A. Posterior cerebral artery. P.Com.A. Posterior communicating artery. P.A. Pericallosal artery. V.A. Vertebral artery. B.A. Basilar artery. The arrows indicate the leptomeningeal branches of the major cerebral arteries.

in the neck. The fall in pressure in the posterior cerebral artery, however, might be sufficient for a greater flow of blood to occur from the ipsilateral anterior cerebral artery into the occluded middle cerebral field.

Vasodilatation following vertebral occlusion may also play a part in the appearance of the increased anterior cerebral collateral flow. Macdonald & Potter (1950) found considerable dilatation of the basilar artery in the rabbit after occlusion of both carotid arteries, and the Russian worker Stenik (1954) has shown also in the rabbit compensatory widening of the

internal carotid, basilar and vertebral arteries at the base of the brain, after occlusion of the carotid and vertebral arteries in the neck. Meyer, Fang & Denny-Brown (1954) showed in the monkey dilatation within the pial circulation after occlusion of a major vessel in the neck. It is therefore possible that the increased anterior cerebral collateral flow which occurred in the present experiments, when the middle cerebral artery was occluded after bilateral vertebral ligation, resulted from dilatation either in the basal vessels of the circle of Willis or in leptomeningeal channels or in both. The alteration in the pattern of collateral flow in the territory of an occluded artery as a result of spasm in the vessels in the immediate vicinity of an occluding clip is readily understandable. In the present experiments, on clipping the middle cerebral artery, spasm of the origin of the ipsilateral anterior cerebral artery was often observed, which would reduce its capacity to take over a proportion of the middle cerebral field. This view is supported by the finding that the time course of development of collateral flow after permanent occlusion of the middle cerebral artery and the disappearance of spasm after its temporary occlusion were of the same order. The collateral flow of ipsilateral anterior cerebral origin developed within half an hour of middle cerebral occlusion, and the arterial spasm produced by temporary middle cerebral occlusion usually disappeared during a similar time. This duration of the spasm and the finding that it was localized to the vicinity of the occluding clip, and varied in intensity, is in agreement with previous descriptions of arterial spasm produced by mechanical stimulation in the pial vessels and basilar artery (Florey, 1925) and in the pial vessels on the surface of the hemisphere (Riser, Meriel & Planques, 1931; Echlin, 1942; Lende, 1960).

The spasm produced in the present experiments was less severe than that observed by Harvey & Rasmussen (1951). In their experiments, spasm was so intense that the artery became 'difficult to find'. Associated with this spasm they observed reduced pulsation in the superficial vessels of the hemisphere and they attributed this reduced pulsation to a peripheral propagation of the spasm; but it may well have resulted merely from the reduced pulse pressure which must have occurred distal to partial occlusion of the main vessel. This explanation appears to be more likely because in the present experiments, as well as in those of Florey (1925), Echlin (1942) and Lende (1960), the area of spasm produced by mechanical stimulation of cerebral vessels was always clearly demarcated.

The demonstration of collateral flow in the lower part of the middle cerebral field from the vertebrobasilar circulation after middle cerebral occlusion indicates the presence of functioning anastomoses between carotid and vertebrobasilar circulations on the surface of the cerebral hemispheres, as well as in the circle of Willis. This finding is in agreement

with the vasodilatation seen in the border zone between middle and posterior cerebral arteries on the occipital pial surface in macaques following carotid or vertebral occlusion in the neck (Meyer *et al.* 1954). From his angiographic studies in human beings, Potter (1959) has drawn attention to the possible importance of such collateral vessels from the basilar circulation in the collateral supply of the field of an occluded middle cerebral artery.

The present findings on perfusion of the macaque brain confirm the view that the perforating branches of the major cerebral vessels have few anastomotic connexions, in contrast to the surface branches of the same vessels. The areas not perfused after middle cerebral occlusion with diathermy obliteration of the central branches were different from those in dogs (Symon, 1960). This is accounted for by the different origin of the anterior choroidal artery in the two species. In the dog, as recorded by Rosomoff (1956), the origin of this vessel is from the proximal few millimetres of the middle cerebral artery, whereas in the majority of subprimates and primates it springs directly from the internal carotid artery (Abbie, 1934).

Shellshear (1920) has pointed out that in primates the perforating branches which supply the claustrum arise separately from the striate branches of the middle cerebral artery, and some way lateral to them. In the present experiments it was often difficult to occlude the claustral branches without coagulation of the first cortical branches of the main vessel, which explains why the claustrum was often perfused.

SUMMARY

1. In the macaque the effect of occlusion of the middle cerebral artery on blood flow in the pial circulation has been examined by means of the technique, previously applied in the dog, of observation of the flow of radioactive red cells through an area of the middle cerebral field.

2. After occlusion of the middle cerebral artery there is persistent blood flow in the middle cerebral field.

3. Only a small proportion of this collateral blood flow comes from the ipsilateral anterior cerebral artery, the main contribution is made from the contralateral carotid circulation via the common pericallosal artery, and, in the lower part of the middle cerebral field, from the vertebrobasilar circulation.

4. The fact that the contribution from the ipsilateral anterior cerebral artery was usually small is explained by two factors: the junction in the macaque of the two anterior cerebral arteries in a common pericallosal artery, and the ready production of vascular spasm in the basal arteries of this species.

5. By a perfusion technique the poor collateral blood supply within the basal ganglia has been confirmed.

6. The findings are compared with those observed in dogs.

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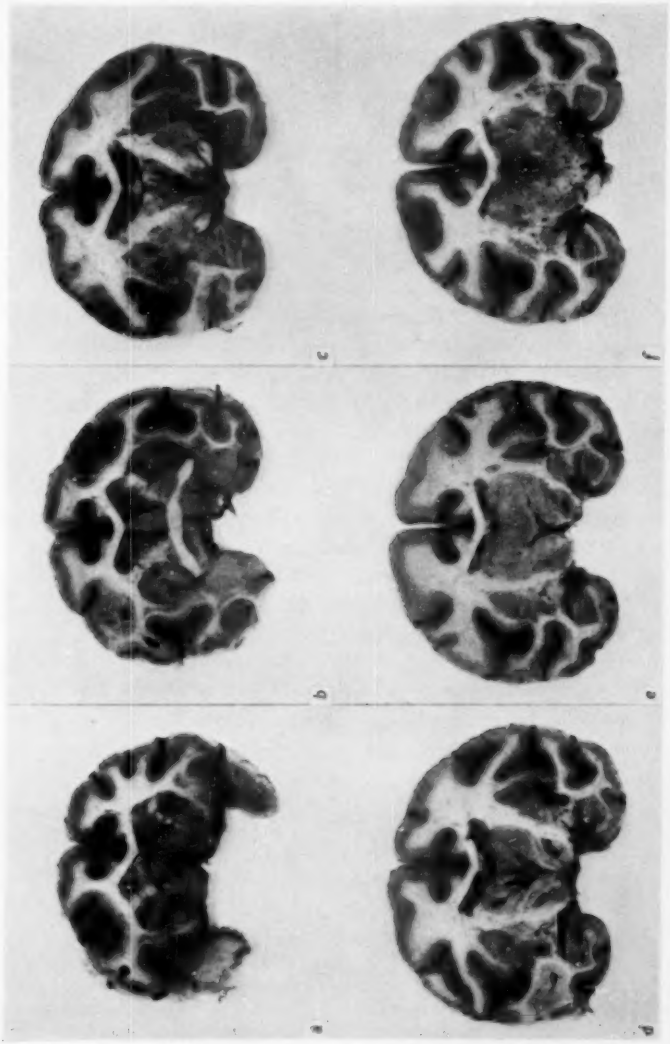
EXPLANATION OF PLATE

PLATE 1

Coronal sections of macaque brain after perfusion with carmine gelatine. In this experiment middle and anterior cerebral arteries were occluded at their origin by silver clips, and the middle cerebral artery occluded by diathermy from its origin to its proximal bifurcation. The area of insertion of the counter is visible in sections *b*, *c* and *d*, above the Sylvian fissure. The blackened areas in the temporal lobe in sections *b–f* are due to operative trauma.



L. SYMON

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THE MECHANISMS BY WHICH DISTENSION OF THE LEFT ATRIUM PRODUCES DIURESIS IN ANAESTHETIZED DOGS

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(*Received* 1 *May* 1961)

Recent reviewers primarily interested in renal (Smith, 1957) and cardiovascular physiology (Neil, 1960) have accepted a theory that distension of the left atrium of the heart sets up afferent impulses in the vagus nerves which decrease the release of antidiuretic hormone from the neurohypophysis, and so cause diuresis. The theory is within the general conception that the renal excretion of water and electrolytes is partly governed by the volume in some fluid compartment of the body. Support for this role of atrial receptors has been derived from the experiments of Henry, Gauer & Reeves (1956), who found that in anaesthetized dogs inflation of a balloon in the left atrium caused an increase in urine flow with a time course and other characteristics which could be accounted for by a diminished release of antidiuretic hormone. The paper of Henry *et al.* (1956) contains little evidence to support this interpretation. In view of the theoretical importance attached to them the experiments have been repeated and extended in this paper; whilst the main results are confirmed, the small and variable size of the response is emphasized and additional observations make untenable the explanation that the diuresis is due to decreased release of antidiuretic hormone. A preliminary account has already been published (Ledsome, Linden & O'Connor, 1961).

METHODS

The experiments were carried out as described by Reeves, Henry & Gauer (1956). Dogs of 10–15 kg were given 15 mg morphine sulphate by subcutaneous injection and 1 hr later were anaesthetized by the intravenous infusion of 1% chloralose (British Drug Houses; 10 ml. = 0·1 g/kg) in sodium chloride solution 0·6 g/100 ml. Subsequently during the experimental procedures a steady state of light anaesthesia was maintained by the infusion every 10 min of either 1 or 0·5% chloralose, about 1 ml./kg. Each ureter was catheterized through a flank incision into the peritoneal cavity, and urine volume was measured every 10 min (Henry *et al.* (1956) collected urine from a urethral catheter). With the animal under positive-pressure ventilation from a Starling 'Ideal' Pump, the chest was opened in the left fifth intercostal space, and a balloon inserted into the left atrium through the appendage in which it was secured by a ligature. The chest was closed, air expelled through a drainage tube in the 7th intercostal space and normal respiration restored. The operative

procedures were completed in about 2½ hr and 2 hr were then allowed to elapse before the first experimental tests. Rectal temperature was maintained between 37·5 and 38·5° C.

In experiments in which one kidney was denervated the right kidney was approached through an incision 1 cm below and parallel to the 12th rib. All fascial connexions were divided between ligatures, until the only attachments of the kidney were the blood vessels and the ureter. Obvious nerve trunks were severed, the vessels were stripped free of fascia and the ureter catheterized and divided below the catheter.

The atrial balloon was made from a 2 cm length of the finger of a surgical glove tied over the end of a polyethylene tube of 2 mm bore. A second polyethylene tube of 1 mm bore was tied alongside the atrial balloon for the measurement of mean left atrial pressure. The balloon was judged to be satisfactorily placed when it lay with its base included in the ligature around the tip of the atrial appendage. It was expanded at each test by 1 ml. saline/kg body weight, or less if the atrial pressure had already risen by about 20 cm H_2O.

Femoral arterial blood pressure and mean left atrial pressure were recorded by capacitance manometers operating ink writers; the frequency response of the channel recording femoral blood pressure was flat (±5%) to 20 c/s. In some experiments respiration was recorded from a manometer connected to a cannula in the right pleural cavity or in the trachea.

Vasopressin was infused through a cannula threaded through a femoral vein so that its tip lay in the inferior vena cava. A motor-driven syringe delivered 0·14 ml. of 0·9% NaCl solution/min to which was added vasopressin (Pitressin; Parke, Davis and Co. batch LZ 1094C or LY 258J) to give the stated rate of infusion. The batches of Pitressin used contained a mixture of lysine vasopressin and arginine vasopressin with the lysine vasopressin probably predominating (personal communication, Parke, Davis and Co.), and were labelled as containing 20 u./ml. of pressor activity. In this paper, 1 m-u. means 0·00005 ml. of Pitressin.

Urine was analysed for sodium and potassium by flame photometry, and for ammonium by the method of Conway (1957). Freezing-point depression was determined by means of a standard Beckmann thermometer graduated to 0·01° C and the osmolar content of the urine was calculated. pH was determined approximately by indicator papers.

RESULTS

Time course and size of the diuresis

Figure 1 shows the result of an experiment similar to those illustrated by Henry *et al.* (1956), in which a balloon was inflated in the left atrium and the inflation maintained for 30 min. In response to the obstruction produced by the balloon the mean pressure in the femoral artery fell 20 mm Hg, there was a rise of 20 cm H_2O in mean left atrial pressure, heart rate rose by about 20 beats/min, and respiration increased by 10 breaths/min. Urine volume from the two kidneys increased gradually from 0·3 ml./min to reach 1·9 ml./min after 30 min.

To present the results of all experiments in which the balloon in the left atrium was inflated for 30 min the results are plotted in Fig. 2 in the form used by Henry *et al.* (1956); the mean rate of urine flow (ml./min) for the 40 min preceding the test and the 40 min following the diuresis (i.e. the mean of urines 1, 2, 3, 4, 9, 10, 11, 12 in Fig. 1) was regarded as the control rate, to be compared with the rate during the diuresis (i.e. mean of urines 6, 7, 8 in Fig. 1). Forty-two balloon inflations were made in

eighteen dogs. Figure 2 shows the results of twenty-four inflations in which the vagus nerves were intact and no infusion of vasopression was made. The continuous line separates two observations in which urine volume (ml./min) decreased, from twenty-two in which there was an increase. The interrupted lines separate nine observations when the urine volume during the diuresis was 1–2 times that during the control period,

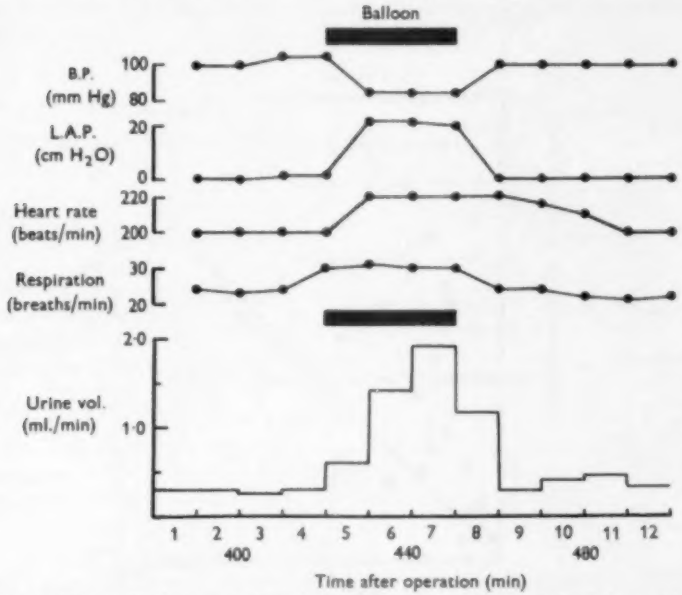

Fig. 1. Effect of inflating a balloon in the left atrium, for the 30 min indicated by the solid bar. From above downwards, femoral arterial pressure, left atrial pressure, heart rate, respiratory rate and urine volume.

six observations 2–3 times, and seven observations more than 3 times the control volume. The scatter is similar to that shown in the corresponding figure of Henry *et al.* (1956). The small size of the diuresis is illustrated by the fact that on only two occasions did the urine volume exceed 2 ml./min compared with 6 ml./min usually attained during water diuresis in the conscious dog.

During control periods mean left atrial pressure was between 0 and 17 cm H_2O and rose during the inflations by 12–32 cm H_2O. Mean arterial blood pressure in the control periods was 75–145 mm Hg and fell during the obstruction of the left atrium by 0–30 mm Hg. The average mean fall in arterial pressure in the twenty-four experiments was 10 mm Hg (S.E.M. $\pm$ 1·3). The heart rate was between 75 and 200 beats/min and increased

during the inflations by 10–120 beats/min, the largest increases being on the occasions when the control rates were low. The respiratory rate was 10–45 breaths/min and was increased by about 15 breaths/min.

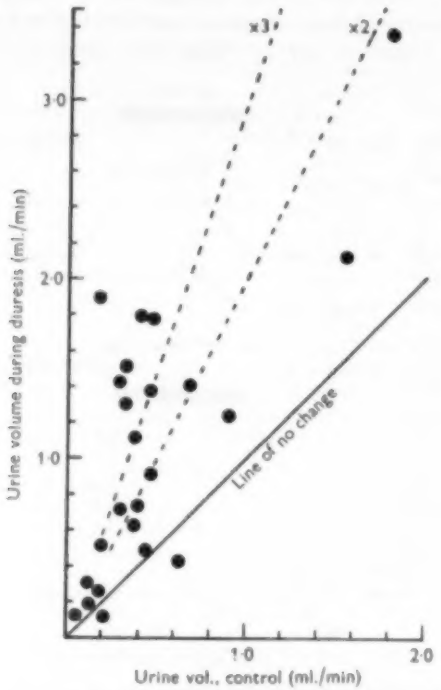

Fig. 2. Results of twenty-four balloon inflations in eighteen dogs. Urine volume during the diuresis compared with urine volume during the control periods. The interrupted lines indicate diuresis of two and three times the control values.

The results in Fig. 2 leave no doubt that inflation of the balloon did produce a diuresis, but detailed examination of the response must be confined to examples where the diuresis was at least twice the resting flow. A smaller response (e.g. increase of urine flow from 0·4 to 0·7 ml./min) could not clearly be recognized against the variations within control periods, and description of its time course or of the changes in urinary composition was impossible.

If inflation of the balloon in the left atrium was maintained for periods longer than 30 min the diuresis began to decrease despite the continued distension of the atrium. Figure 3 shows an experiment in which a balloon was inflated for 70 min. Urine volume increased to reach a maximum

after 50 min and thereafter decreased, although the high left atrial pressure, low arterial blood pressure and raised heart and respiratory rates were maintained. This was the most prolonged diuresis observed and on other occasions the diuresis began to fall off after 30–40 min; Henry *et al.* (1956) show a similar record. Thus Fig. 1 could be misleading in that it might give the impression that obstruction of the left atrium caused the urine flow to rise to a plateau which would be maintained until the balloon was deflated.

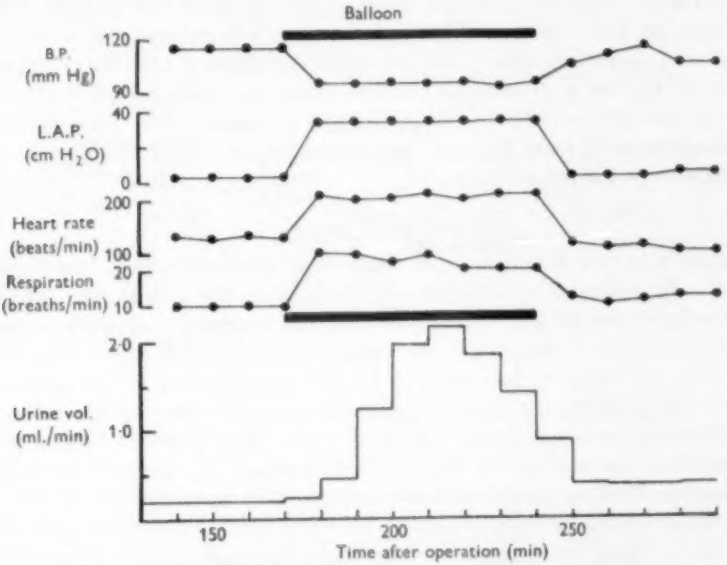

Fig. 3. Effect of inflating a balloon in the left atrium for 70 min; conventions as in Fig. 1.

To obtain a diuretic response to inflation of the balloon, Henry *et al.* (1956) thought it necessary to have the chest closed and the animal breathing spontaneously, and this technique was followed in the experiments so far described. However, in each of four experiments in which the chest was left open and respiration maintained by positive-pressure ventilation, a diuretic response was obtained. Figure 7 shows such an experiment in which there were typical changes in arterial pressure, left atrial pressure, and heart rate, and a diuresis of four times the control level, artificial respiration being maintained constant at 18/min throughout.

Henry *et al.* (1956) stated that at least 2 hr should elapse after the operation before inflating the balloon, and usually we followed their practice.

However, diuresis has been observed on each of two occasions when a balloon was inflated within an hour of the operation; Fig. 7 illustrates this finding, the diuresis being produced 40–70 min after completion of the operation.

Infusions of dog blood, plasma or dextran (6 g/100 ml. in NaCl solution 0·9 g/100 ml.; 'Dextraven', Benger Laboratories Ltd.) were occasionally given during the experiments as supportive measures without enhancement of the diuretic response to balloon inflation, although Henry *et al.* (1956) claimed that infusions of bovine albumin or dog blood did improve the response. However, transient increases in urine volume occurred without inflation of the balloon after seven out of eleven infusions, as in the example in Fig. 6. On three occasions a diuresis occurred spontaneously without obvious association with any experimental procedure. The time course and magnitude of these diureses was similar to the effects of inflation of the balloon in the left atrium.

Excretion of solutes

Figure 4 shows the results of analysis of the urine from the right kidney. In response to inflation of a balloon in the left atrium, urine volume increased from 0·12 to 0·8 ml./min, an increase of about 6 times, whilst during the same period the excretion of the solid constituents in the urine increased by less than 70%; the concentration of the various solutes fell to about one fifth of the control values. Analysis for sodium and potassium was made in nine tests where the diuresis was 2–6 times the control flow and showed similar small changes in the excretion of these substances. Total urine solutes were estimated by means of the freezing-point depression in five tests in which the urine volume during the diuresis rose to 3·5 times the control volume, whilst the excretion of the solutes only increased by 20%. The pH in the control periods was about 7·0, and was not altered during the diuresis; increases in ammonium excretion were small in two experiments where this was examined.

Effect of infusion of vasopressin

Figure 5 shows the effect of inflation of a balloon in the left atrium by exactly the usual procedure, but during an infusion of vasopressin. Infusion at a rate of 0·025 m-u./kg/min started 50 min before the test and continued until 30 min afterwards had no effect on urine volume and in no way modified the diuretic response, which was similar to the diureses in Fig. 5 when no vasopressin was infused. A diuretic response was observed on each of five occasions when a balloon was inflated during the infusion of 0·025 m-u./kg/min. Larger infusions of vasopressin of up to 0·1 m-u./kg/min were also used. Figure 6 depicts the effects produced by two

balloon inflations made during infusion of vasopressin at a rate of 0·1 m-u./kg/min. On each occasion a diuresis of about twice the control volume was obtained. Infusion at this rate was without obvious effect on control urine volume, left atrial pressure or arterial blood pressure.

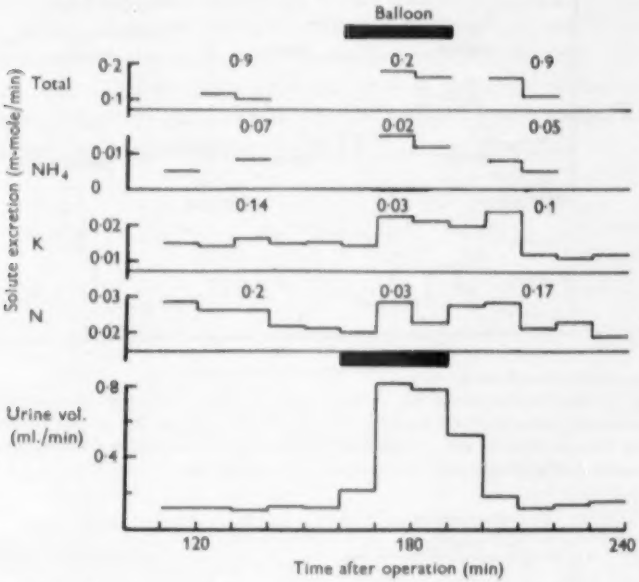

Fig. 4. Composition of urine and rates of excretion from right kidney during inflation of a balloon in the left atrium. From above downwards, urine solute excretion—total solute, ammonium, potassium and sodium—and urine volume (ml./min). Figures written above each line show urine concentration (M).

Since it was uncertain whether the batches of Pitressin contained lysine or arginine vasopressin, they were tested on a conscious dog. Infusion at 0·009 m-u./kg/min was begun and 400 ml. of water given by stomach tube 5–10 min later. In the absence of vasopressin water diuresis always resulted but 0·009 m-u./kg/min of either batch of Pitressin was adequate to prevent the diuresis until after the infusion was ended. Thus the rate of infusion of vasopressin in Fig. 5 was at least 2·5 times and in Fig. 6 was 10 times the rate needed to abolish water diuresis in the conscious dog.

Effect of vagotomy

In eight tests on five dogs a balloon was inflated in the left atrium after section of the vagus nerves in the neck and no diuresis resulted in any of these tests (Fig. 7). In five of the tests there was a small fall in urine flow.

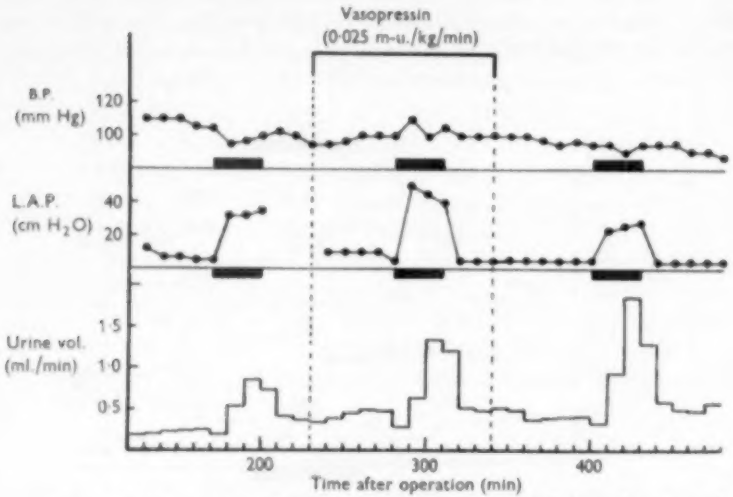

Fig. 5. Diuresis produced by balloon inflation during infusion of vasopressin. Vasopressin infusion 0·025 m-u./kg/min was started 50 min before and continued until 30 min after the second inflation. From above downwards, femoral arterial pressure, left atrial pressure and urine volume.

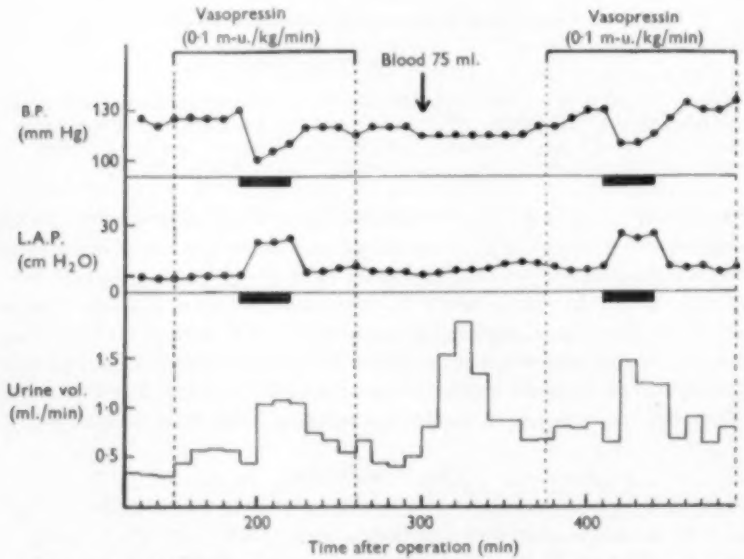

Fig. 6. Two balloon inflations during infusion of vasopressin 0·1 m-u./kg/min. Conventions as in Fig. 5. A diuresis following an infusion of 75 ml. of dog blood is also shown.

The balloon was inflated at times varying from 40 min to 4 hr after vagotomy; in two experiments the vagi were cut immediately after the operation, whereas in three (e.g. Fig. 7) a typical diuresis was elicited in a test inflation before the vagi were cut. When the dog was breathing naturally vagotomy caused changes in the rate and depth of respiration; in Fig. 7 the experiment was under positive-pressure artificial ventilation which was maintained unchanged throughout. Heart rate was high after vagotomy and did not change when the balloon was inflated, but changes in left atrial pressure and femoral arterial pressure were similar to those seen during the test before vagotomy.

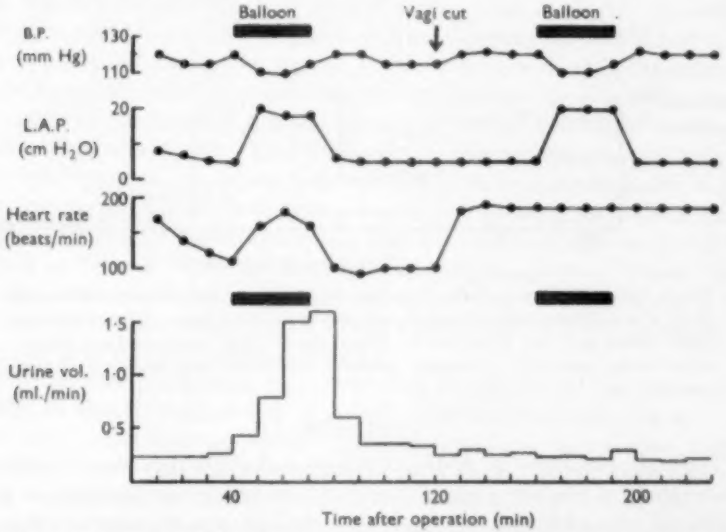

Fig. 7. The effect of balloon inflation before and after section of the vagus nerves. From above downwards, femoral arterial pressure, left atrial pressure, heart rate and urine volume. In this animal the chest was open and positive-pressure artificial ventilation was maintained unchanged throughout. Vagi cut at time marked by the arrow.

Effect of denervation of a kidney

The right kidney was denervated in four dogs and 3–5 hr later, when urine flow from both kidneys was steady, a balloon was inflated in the left atrium. Figure 8 shows the result of one experiment. During the control period urine volume from the denervated kidney was about 50% greater than from the intact kidney, and when the balloon was inflated the urine volume doubled in both kidneys. The typical diuresis was also produced in a denervated kidney in each of the three other experiments.

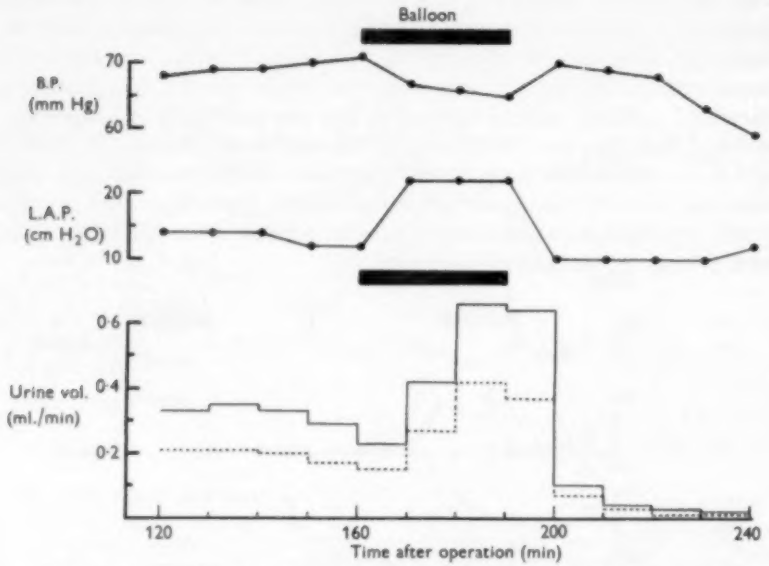

Fig. 8. Effect of balloon inflation on a denervated kidney. From above downwards, femoral arterial pressure, left atrial pressure and urine volume. —— urine volume from denervated (R) kidney; ----- urine volume from innervated (L) kidney. Chest open and positive-pressure artificial ventilation maintained unchanged throughout.

DISCUSSION

The main experimental finding of Henry *et al.* (1956) has been confirmed; obstruction of the left atrium in dogs under chloralose anaesthesia produced an increased flow of urine. The diuresis was transient, and small when compared to water diuresis in conscious dogs, and it was never possible to predict from the heart and respiration rates, arterial and left atrial pressures recorded during the control periods whether the effect of inflating the balloon would be comparatively large or virtually absent. Technical procedures mentioned by Henry *et al.* (1956) as likely to ensure a good response proved unreliable and no way was found of obtaining consistent responses. However, despite the occasional spontaneous diuresis the results in Fig. 2 were sufficient to establish that inflation of the balloon *did* produce a diuresis, but its size and variability made detailed investigations difficult and attempts to explain the mechanisms involved must be largely speculative.

Henry *et al.* (1956) and Henry & Pearce (1956) suggest that the first stage in the mechanism is the stimulation of receptors in the left atrium.

The existence of receptors stimulated by distension of the intrathoracic parts of the circulation is now well established, and in the dog they have been found in the pulmonary arteries (Coleridge & Kidd, 1960) and at the junctions of the venae cavae and pulmonary veins with the atria (Coleridge, Hemingway, Holmes & Linden, 1957). Receptors in the pulmonary arteries and left atrium presumably discharged more frequently when a balloon was inflated to obstruct the mitral opening; Henry & Pearce (1956) recorded the discharge from a receptor in the left atrium. Also Henry & Pearce (1956) observed no diuresis when the left atrium was obstructed with the vagi blocked by cold, and we were unable to produce a diuretic response to balloon inflation after vagal section (Fig. 7). However, section of the vagi involves the interruption of many different efferent and afferent nerve fibres and cannot be regarded as merely interrupting the afferent limb of a specific reflex. Section of the vagi produces a very great disturbance of circulation and respiration and absence of the diuresis might be due to such general effects rather than the loss of the afferent impulses from intrathoracic receptors. Since we could not define circumstances under which the response appeared, it is impossible to say whether the general state of the animal after vagotomy was or was not such that diuresis would be expected. Thus it is possible that increased discharge from intrathoracic receptors is a necessary part of the sequence by which obstruction of the left atrium produces diuresis, but no real evidence either for or against such a hypothesis has yet been presented.

Whether the mechanism is a reflex with its afferent fibres in the vagi or the diuresis is produced by some other mechanism, some agent must eventually reach and act on the kidney to produce the increase in the urine flow. Two possibilities appear to be excluded by our experiments. First, the diuresis occurred in the denervated kidney (Fig. 8) and therefore was not due to changes in the activity of the renal nerves. Secondly, the diuresis occurred during infusions of vasopressin (Figs. 5, 6), and therefore was not due to decreased release of antidiuretic hormone of the neurohypophysis. The characteristics of the urine in Fig. 4 do not match the effects of any of the agents known to have important effects on the volume and composition of the urine (O'Connor, 1961). If the diuresis were due to dilution of plasma protein or increased renal blood flow, a greater increase in sodium excretion would be expected. If it were an osmotic diuresis the excretion of osmotically active solutes should increase nearly in proportion to the increase in urine volume. If a large amount of an organic anion entered the plasma and was excreted in the urine then the urine would become acid and contain ammonium. The time course and nature of the diuresis is unlike any effect to be expected from an altered rate of release of adrenal cortical steroids. The usual effect of increased

release of adrenaline or noradrenaline is decreased urine flow and decreased excretion of sodium (O'Connor, 1961). Thus the agent acting on the kidney to produce the effect of Fig. 4 cannot be recognized by the characteristics of the urine. However, it should be realized that the diuresis in the experiments of this paper was small, transient and in animals under chloralose anaesthesia subjected to a severe operation. The urinary effects of the agents known to act on the kidney (O'Connor, 1961) have been described from experiments in which large effects persist in conscious animals; comparison between the two types of experiments may be misleading.

When the experiments of Henry *et al.* (1956) are cited in the literature on volume receptors, it is usually assumed that the diuresis is due to a decreased release of antidiuretic hormone from the neurohypophysis. Henry *et al.* (1956) offered no evidence for this suggestion, which is based on a comparison with a somewhat similar diuresis produced by breathing from a reservoir containing air at pressure less than atmospheric (Gauer, Henry, Sieker & Wendt, 1954; Sieker, Gauer & Henry, 1954). Our finding that the diuresis occurs during infusion of vasopressin is in obvious conflict with the accepted explanation, and a detailed discussion of the evidence concerning the activity of the neurohypophysis in dogs under chloralose anaesthesia therefore seems necessary.

Theobald (1934) gave to dogs by stomach tube anaesthetic doses of chloralose dissolved in 300 ml. of water. The animals 'fell asleep' during the course of the water diuresis and the diuresis could be inhibited by intravenous injection of the same doses of vasopressin as inhibit water diuresis in the conscious animal. Apparently immediately after induction of chloralose anaesthesia there is no abnormal release of antidiuretic hormone from the neurohypophysis, and the kidney tubules respond normally to vasopressin. The conditions in our experiments were very different in that chloralose anaesthesia had been maintained for at least 3 hr and a major surgical operation carried out. Water, injected intravenously or given by stomach tube to these animals, did not produce water diuresis and Verney (1929) found that under similar conditions water was absorbed from the stomach but no diuresis resulted. In experiments like those of this paper Baïsset, Douste-Blazy, Montastruc & Valdiguié (1957) describe antidiuretic activity in extracts of jugular-vein blood, assayed as equal to 0·4 m-u. vasopressin/ml. serum, which would indicate the release of about 40 m-u./min, as compared to the release in normal conscious dogs of about 0·1 m-u./min (Verney, 1947): premedication with morphine might cause some release of antidiuretic hormone (Duke, Pickford & Watt, 1951). Eisen & Lewis (1954) indicate that antidiuretic hormone may be released during operations in human subjects and it was for this reason that Henry *et al.* (1956) waited 2–4 hr after completion of

the operation before testing the effect of inflating the balloon in the left atrium; we have obtained the diuresis only 40 min after the operation (Fig. 7). It is not clear whether water diuresis fails to occur under these conditions because of a continual release of antidiuretic hormone or some other substance or because of some defect in the kidney itself. Chloralose in conjugated form is excreted in the urine (Kochman, 1923) and may well affect the tubular cells; in our experiments the urine contained a reducing substance which, with Benedict's quantitative reagent, did not give the colour change characteristic of glucose. Since water diuresis cannot be established under the conditions of our experiments it is impossible to test whether the kidneys respond normally to injections of vasopressin and there is no standard by which to assess the effective dose. The diuresis caused by obstruction of the left atrium occurred during infusion of vasopressin at 10 times the rate needed to stop water diuresis in conscious animals and is therefore not produced by any *normal* function of the neurohypophysis. If the neurophypophysis is part of the mechanism producing diuresis in the experiments in Figs. 5 and 6, the sensitivity of the kidney to antidiuretic hormone must also be abnormal, and the effect is so far removed from normal function of the neurohypophysis as to have no relevance in the study of the normal control of urinary volume.

The experiments of Henry *et al.* (1956) are not usually cited as evidence for the general theory of the control of urinary volume by volume receptors. Henry *et al.* (1956) carried out their experiments because study of the effects in dogs and man of breathing from a reservoir at pressure less than atmospheric had convinced them that intrathoracic volume receptors did produce inhibition of the neurohypophysis and so diuresis, and their object was to determine the site of the receptors. Discussion in this paper of the experiments of Henry *et al.* (1956) certainly indicates that they can have no general application until the causative mechanisms can be elucidated, and they should not be used in interpreting the effects of negative-pressure ventilation or other procedures which affect urine flow. Likewise this paper provides no direct evidence which denies either the general conception of volume receptors or the interpretation of the effects of negative-pressure ventilation as due to inhibition of the neurohypophysis; these ideas can be criticized on other grounds (O'Connor, 1961).

SUMMARY

1. In confirmation of the findings of Henry *et al.* (1956), distension of the left atrium of dogs under light chloralose anaesthesia produced a diuresis which was small, transient and variable in size.

2. During the diuresis there was at most a small increase in the excretion of sodium, potassium or total urinary solutes.

3. Diuresis was produced on balloon inflation after renal denervation.

4. The diuresis was also produced during infusion of vasopressin at a rate adequate to inhibit water diuresis in the conscious dog.

5. The diuresis did not occur after section of the vagus nerves.

6. The mechanisms involved cannot be satisfactorily explained.

We wish to thank Mr J. Brook for his excellent technical assistance.

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THE RETURN OF BLOOD FLOW TO FINGERS IN ICE-WATER AFTER SUPPRESSION BY ADRENALINE OR NORADRENALINE

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Cold vasodilatation, the appearance of a high blood flow in a part of the body some minutes after it has been cooled to near freezing-point, has been attributed to axon reflexes alone (Lewis, 1930; Celander & Folkow, 1953). However, Greenfield, Shepherd & Whelan (1951) observed the dilatation in denervated fingers and Duff, Greenfield, Shepherd, Thompson & Whelan (1953) failed to obtain evidence that acetylcholine or histamine were responsible for it. The fact that isolated arteries ceased to respond to constrictor drugs when cooled to near freezing-point (Keatinge, 1958) suggested that a direct effect of low temperature on blood vessels might be a major cause of cold vasodilatation. The present experiments were therefore made to show whether the resistance blood vessels of human fingers, after being constricted by adrenaline or noradrenaline when warm, would maintain the constriction when cooled to 0° C. A preliminary communication of these findings has been published (Keatinge, 1961).

METHODS

Subjects and experimental conditions. The subjects were healthy men and women, aged between 18 and 32, who volunteered for the experiments. All experiments were made on index fingers, the left always being used for iontophoresis and the right as the control. They were made in a room maintained either at 31–32 or 33–34° C by thermostatically controlled electric heating elements fitted to a fan. Humidity was kept high by a tank of water (surface area 3·5 m^2) at 40° C in the room.

Procedure. The subject sat for 20 min in the experimental room, after which measurements of heat flow were generally made on both index fingers in water at 30° C. One or more periods of iontophoresis were then carried out, each followed by a determination of heat flow with the finger in water at 30° C. The fingers were then dipped into water at 37° C for 6 min to bring them both to the same temperature, and were then dipped up to the middle of the proximal phalanx in a mixture of ice and water which was stirred mechanically in an insulated circular container to keep it flowing steadily past the fingers. During the immersions in ice-water, readings of heat flow were made every 30 sec until the flow returned and every 60 sec thereafter. Further readings were made in water at 30° C in a number of subjects. In all experiments the subjects were seated with their hands 40–45 cm below the sternal angle.

Iontophoresis. The solution of adrenaline used for iontophoresis contained adrenaline free base (British Drug Houses, Ltd.) 4 %, titrated to pH 4·5 against phosphoric acid. It was made up by weighing 2 g of adrenaline, suspending this in 46 ml. of distilled water, and running in 20 % phosphoric acid at a room temperature of 22–24° C until the adrenaline had dissolved. Its pH, measured by a Cambridge Instrument Company pH meter (Portable Bench Type) and a glass electrode, was then adjusted to 4·5 ± 0·1 by running in first 20 % and then 5 % phosphoric acid. The final solution was found to contain less than 0·05 m-equiv of Na^+ or Cl^- ions and less than 0·005 m-equiv of K^+. The solution of noradrenaline contained noradrenaline bitartrate ('Levophed', Bayer) 4 % and was made by dissolving 2 g of the drug in 48 ml. of distilled water. It had a pH of approximately 3·15.

During iontophoresis the left index finger, previously washed with alcohol, soap and water, was dipped up to the middle of the second phalanx into the solution, which was at room temperature. An electrode was placed in contact with the left forearm through a pad of cotton wool soaked in saline, and a current was passed from the solution to the electrode. This current was adjusted to 2 mA by tapping an e.m.f. from a 75 V dry cell by a slide resistance. The current was raised to its full value in 10–15 sec at the beginning of each period of iontophoresis, and lowered to zero in the same time at the end. A rubber tourniquet was clipped round the base of the finger during the first 20 min of iontophoresis on each occasion to prevent the drug from being carried away rapidly during this time, before the blood flow was largely suppressed by the drug. The surface area of the part of W. R. K's finger exposed to iontophoresis was measured by rolling it on graph paper and was found to be 16 cm^2 giving a current density of 0·125 mA/cm^2. The surface area of his forearm, between the elbow and wrist, was found to be 415 cm^2.

Blood flow was assessed by individually calibrated heat-flow disks (Hatfield, 1950). One was stuck by Nobecutane (Evans) to the pad of each index finger and supported by a rubber band. The e.m.f. from each was led to a switch by which each could be connected through a resistance to a galvanometer. Rates of heat flow are expressed in relation to the temperature difference between the water in which the finger was dipped and the sublingual temperature, in order to facilitate comparison of results obtained at different water temperatures. The galvanometer could be read to 0·1 mm; this represented a heat flow of approximately 0·0002 cal. cm^{-2}. min^{-1}. $°C^{-1}$ temperature gradient with the finger in water at 30° C and approximately 0·00004 cal. cm^{-2}. min^{-1}. $°C^{-1}$ with the finger in water at 0° C. When low flows were measured the zero position of the galvanometer was checked before every reading by breaking the connexion between disk and galvanometer briefly with a 2-pole switch. To measure some of the higher heat flows reached by control fingers in ice-water it was necessary to reduce the sensitivity of the system by switching in an additional series resistance. Sublingual temperature was measured by a mercury thermometer accurate to 0·05° C which was kept under the tongue, with the mouth closed, for 90 sec before taking a reading. The sublingual reading used in this calculation was taken within 1–2 min of heat-flow readings from fingers in warm water, but often at longer periods from the repeated readings taken in ice-water, when the gradient was large and small changes in sublingual temperature were of correspondingly little importance in assessing heat flow per degree centigrade temperature gradient. Except in ice-water immersions, when readings were taken at short intervals, heat-flow readings were taken only when a steady rate of heat flow had been reached, which was generally 8–10 min after the finger was dipped into water at a given temperature.

RESULTS

Figure 1 shows the effect of iontophoresis with adrenaline on the blood flow in a man's finger. Before iontophoresis the rates of heat loss from his right and left index fingers in water at 30° C were very similar. Ionto-

phoresis for 10 min reduced the blood flow in his left index finger by more than a half, but further iontophoresis reduced it more slowly, a total of 70 min of iontophoresis being required to reduce the flow to a negligible level. This iontophoresis to the left index finger also slightly reduced the flow in the right. Radiant heat directed on the subject for 10 min, at the end of which he was sweating profusely, did not restore any substantial flow to the left index finger, although it slightly increased the flow in the right.

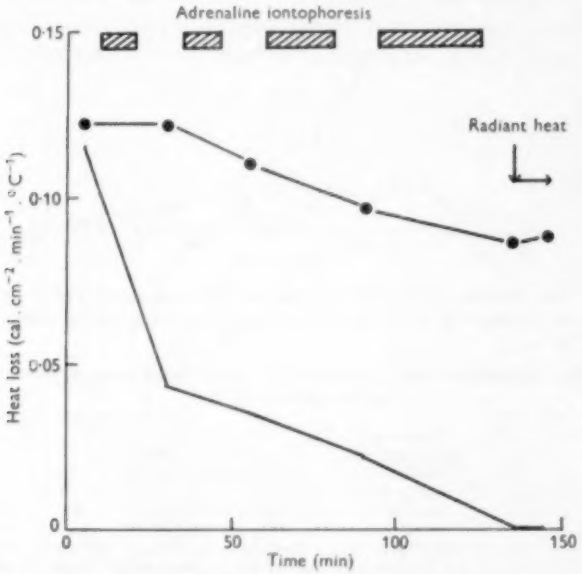

Fig. 1. The effect of iontophoresis of adrenaline on finger blood flow. Room at 33–34° C, finger in water at 30° C. Subject W.R.K. — Finger exposed to iontophoresis; ●—● control finger.

Figure 2 shows the effect of iontophoresis with noradrenaline on the blood flow in another subject's finger. Seventy minutes iontophoresis to the left finger was required to reduce the blood flow in it to a low level, and the procedure only slightly reduced the blood flow in the right index finger. Radiant heat again failed to cause any substantial return of blood flow to the finger exposed to iontophoresis.

Table 1 summarizes the results of fourteen such experiments. In eight of them heat flow in the experimental finger was reduced by iontophoresis to a really low level, arbitrarily set at 0·003 cal.cm^{-2}.min^{-1}.° C^{-1}. At the end of iontophoresis in these eight experiments the mean flow in the left index finger was only 0·0008 cal.cm^{-2}.min^{-1}.° C^{-1}, less than 1 % of

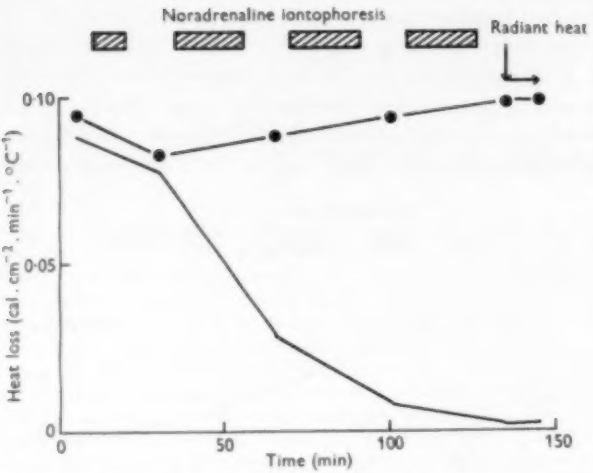

Fig. 2. The effect of iontophoresis of noradrenaline on finger blood flow. Room at 33–34° C, finger in water at 30° C. Subject J.A. Symbols as in Fig. 1.

TABLE 1. Effect of iontophoresis on blood flow in index finger; iontophoresis current 2 mA; finger in water at 30 °C

Subject	Sex	Drug	Period of iontophoresis needed to reduce heat loss below 0·003 cal. $cm^{-2}.min^{-1}.°C^{-1}$ temp. gradient (min)	Total period of iontophoresis (min)	After completion of iontophoresis: Heat loss ($cal.cm^{-2}.min^{-1}.°C^{-1}$) Finger subjected to iontophoresis (left)	Control finger (right)	Sublingual temperature (° C)	Room temperature (° C)
A.M.	M	Adrenaline	Not known	70	0·018	0·060	37·35	31–32
J.A.	F		10–30	70	0·0011	0·079	37·35	
R.B.	M		Not known	70	0·027	0·110	37·20	
J.S.	F		10–30	70	0·0002	0·066	37·40	
P.H.	F		Not known	25	0·012	0·098	37·65	
R.J.	M		Not known	25	0·045	0·106	37·10	
D.S.	M	Adrenaline	Not known	25	0·072	0·091	37·30	33–34
M.W.	F		5–25	25	0·0011	0·089	37·70	
M.S.	F		10–20	20	0·0004	0·092	37·35	
J.H.	M		30–45	45	0·0009	0·100	37·45	
W.R.K.	M		40–70	70	0·0004	0·098	37·35	
J.A.	F	Noradrenaline	50–70	70	0·0021	0·100	37·70	
S.P.	F		Not known	95	0·010	0·063	36·80	
W.R.K.	M		70–90	90	0·000	0·120	37·30	
Mean of the 8 experiments in which heat flow was reduced below 0·003 $cal.cm^{-2}.min^{-1}.°C^{-1}$ by iontophoresis			38	57·5	0·0008	0·093	37·50	

the flow in the control fingers. In the case of adrenaline, 30 min or less of iontophoresis was sufficient to reduce the flow below 0·003 cal.cm^{-2}.min^{-1}.° C^{-1} in four women out of five, but in two men 30–70 min iontophoresis was required to suppress the flow and in two others 70 min iontophoresis was insufficient. With noradrenaline, a longer period of iontophoresis was required to suppress the flow in J.A.'s and W.R.K.'s fingers than was needed with adrenaline. When the flow had been reduced below 0·003 cal.cm^{-2}.min^{-1}.° C^{-1} by iontophoresis with either drug, radiant heat directed on the subject for 10–30 min never raised the flow in the finger exposed to iontophoresis to more than this level.

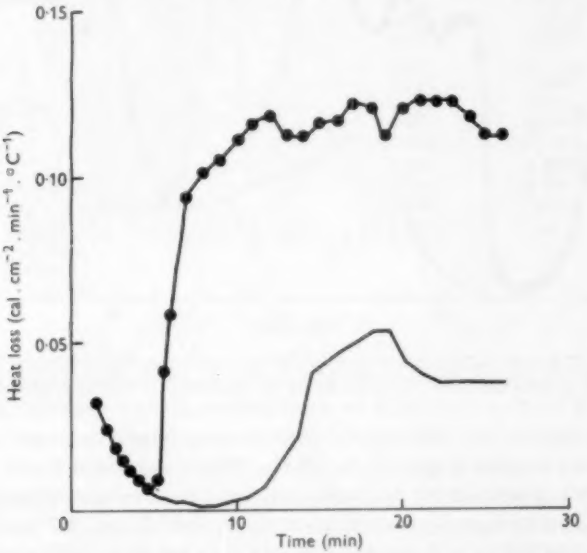

Fig. 3. The return of blood flow to finger in ice-water, after suppression by iontophoresis of adrenaline. Room at 33–34° C. Subject W.R.K. Symbols as in Fig. 1.

Tremor, weakness, and pounding of the heart were generally observed after 30 min iontophoresis with either drug, and were always observed if the iontophoresis was more prolonged. In the finger there was always tingling and some pain during iontophoresis. The pain became worse as iontophoresis proceeded, but was never really severe.

Figure 3 shows the effect of immersing a man's index fingers in ice-water after the flow in one of them had been suppressed by adrenaline. For 5 min after they were immersed the heat loss from both fingers fell off steadily; it then rose abruptly to a high level in the control finger and remained high. In the finger exposed to iontophoresis the heat loss

continued to fall for a further 3·5 min, when it began to rise. It rose more slowly than that of the control finger had done, reached about half the highest level reached by the control finger, and then fell to, and became stabilized at, about a third of the level of the control finger. Figure 4 shows that a similar pattern of flow developed in a woman's fingers in ice-water after the flow in one of them had been suppressed by noradrenaline. The only difference was that the blood flow in her control finger twice fell sharply, though briefly, after it had reached a high level.

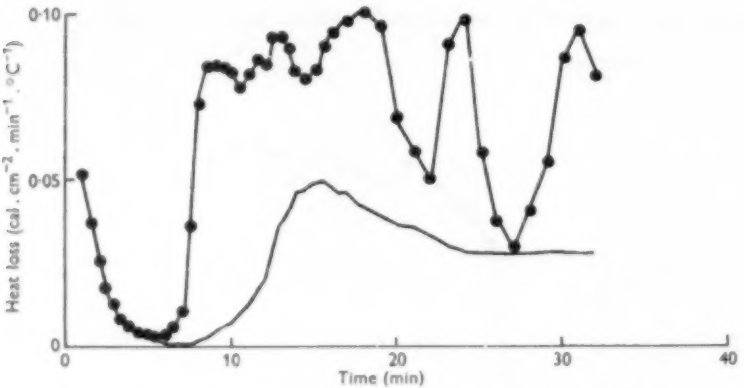

Fig. 4. The return of blood flow to finger in ice-water after suppression by iontophoresis of noradrenaline. Room at 33–34° C. Subject J.A. Symbols as in Fig. 1.

Table 2 summarizes the results of immersing fingers exposed to iontophoresis and control fingers in ice-water. When the blood flow had been adequately suppressed by iontophoresis it always returned later in the finger exposed to iontophoresis than in the control finger, 3·7 min later on average. A high flow always returned in ice-water, 40 % of the control on average, except in J.S. in whom the flow still rose tenfold as compared to the flow at 30° C. The patterns of response shown in Figs. 3 and 4 for fingers exposed to iontophoresis and control fingers were followed closely by 6 of the 8 subjects in whom the flow had been adequately suppressed. The two exceptions were the adrenaline experiments on J.A. and J.S., in which the flow in the fingers exposed to iontophoresis fluctuated slowly up and down in ice-water instead of becoming stabilized; these were the only experiments in which iontophoresis had been continued substantially longer (Table 1) than was required to suppress the flow.

Both fingers exposed to iontophoresis and control fingers became very painful when they were immersed in ice-water. The pain then decreased and, in fingers exposed to iontophoresis in which flow had been suppressed,

TABLE 2. Return of blood flow to index fingers immersed in ice-water after iontophoresis

Subject	Drug	Onset of vasodilatation (min after immersion)		Highest flow reached (cal.cm^{-2}.min^{-1}.° C^{-1} temp. gradient)		Room temperature (° C)
		Finger subjected to iontophoresis	Control finger	Finger subjected to iontophoresis	Control finger	
A.M.	Adrenaline	9·0	2·0	0·036	0·107	31–32
J.A.		7·0	6·5	0·029	0·070	
R.B.		9·0	7·5	0·050	0·127	
J.S.		10·5	1·5	0·002	0·047	
P.H.		5·0	2·5	0·047	0·090	
R.J.		6·5	3·5	0·020	0·093	
D.S.	Adrenaline	9·0	9·0	0·065	0·097	33–34
M.W.		8·0	5·0	0·027	0·098	
M.S.		11·0	3·5	0·010	0·093	
J.H.		7·5	7·0	0·054	0·094	
W.R.K.		8·5	5·0	0·055	0·122	
J.A.	Noradrenaline	8·0	6·0	0·049	0·103	
S.P.		8·0	3·5	0·048	0·060	
W.R.K.		8·0	—	0·059	—	
Mean of the 8 experiments in which flow had been reduced below 0·003 cal. cm^{-2}.min^{-1}.° C by iontophoresis*		8·6	4·9	0·036	0·90	—

* See Table 1.

pain had disappeared by the time the blood flow began to return. The finger at this time was insensitive both to touch and to hard pressure. As the flow returned, the pain reappeared for a time, and receded or ceased when a steady flow was reached. The finger generally became painful again for a few minutes when it was removed from the ice-water and re-warmed. A similar pattern of sensory changes was observed in control fingers, except that sensation in them had generally not disappeared before blood flow returned in the ice-water.

Heat-flow readings in water at 30° C were made on the fingers of some subjects 10 min after the fingers were removed from ice water. In adrenaline experiments the heat flow from the index fingers of J.A. J.S. and M.W. at this time were all less than 0·003 cal.cm^{-2}.min^{-1}.° C^{-1}. In the noradrenaline experiment on W.R.K.'s finger the heat flow was 0·006 cal.cm^{-2}.min^{-1}.° C^{-1} at this time. In adrenaline experiments fingers always remained cold and numb for 12–24 hr after iontophoresis and then recovered slowly. After noradrenaline the coldness and numbness were less marked and largely wore off in 1–2 hr. The numbness at the tip of the finger, which was severe after adrenaline, could be relieved temporarily by swinging the arm vigorously.

DISCUSSION

The blood vessels in these fingers dilated when they were cooled to near freezing-point, even in the presence of enough adrenaline or noradrenaline to suppress the blood flow at higher temperatures. Since sensation had disappeared from the fingers exposed to iontophoresis before the blood flow returned to them, their sensory nerves had apparently ceased to function. The dilatation is therefore unlikely to have been initiated by axon reflexes. The fact that blood flow returned under these conditions, in the presence of the constrictor hormones, supports the view that the response of the resistance vessels of the finger to the hormones was impaired or lost at near freezing-point. The impairment may have been severe in spite of the fact that the peak flows reached by fingers exposed to iontophoresis in ice-water were less than those reached by control fingers; the deeper vessels in them must have been warmed and would regain their ability to respond as the blood flow returned. The earlier observation (Keatinge, 1957) that intramuscular injections of adrenaline failed to suppress cold vasodilatation in the fingers of generally chilled men also suggested that human digital blood vessels responded little if at all, to adrenaline at near 0° C. However, it was uncertain whether adrenaline then reached the digital vessels in effective quantities; there was no doubt that the drugs did reach the vessels, and remain there, in the present experiments.

Although isolated arteries (Keatinge, 1958) ceased to respond to adrenaline as soon as they were cooled, they maintained for a time any contraction that was present at the time they were cooled. Similar behaviour by resistance vessels can explain why cold vasodilatation does not take place immediately after a finger is cooled, and why iontophoresis increased the delay. Isolated arteries regained their power of responding to adrenaline as soon as they were rewarmed, but they regained it incompletely at first. Similar behaviour by the digital resistance vessels could explain why blood flow in fingers exposed to iontophoresis first rose to a high level in ice-water and then became stabilized at a rather lower level. Axon reflexes probably also played a part in these changes after the initial dilatation had raised the finger temperature. Vasodilator substances may also be released by cold independently of nerves, but on present evidence there seems no need to postulate such a release.

The low and fluctuating blood flows sometimes seen during cold vasodilatation in the fingers of generally chilled men (Keatinge, 1957) and after prolonged iontophoresis in two subjects in the present experiments, may have been determined by contraction of the large arteries of the hand and arm. The radial pulse was always weaker after the men were chilled in the former experiments than before they were chilled, and in dogs the large

arteries can contract sufficiently (Davis & Hamilton, 1959) to arrest the circulation to a paw. Arteries often undergo rhythmical contraction (Grant, 1930) when stimulated by adrenaline. The abrupt falls in blood flow, which were often seen in control fingers in ice-water after a high flow had been present for some minutes, were probably started reflexly by minor stimuli; at the time they took place the vessels apparently had been warmed to a high enough temperature and for a long enough time to recover much of their normal contractile power. It was shown earlier (Keatinge, 1957) that vasoconstrictor reflexes will cause abrupt constriction during cold vasodilatation in the fingers of generally warm subjects.

Two points about the effect of iontophoresis in warm fingers need comment. The longer time needed to suppress the flow in men's than in women's fingers can be explained in part, at least, by the smaller size of the women's fingers, and the smaller and briefer action of noradrenaline than adrenaline was presumably due to more rapid breakdown.

The heat flow recorded from fingers in a steady state of heat loss must be due to cutaneous blood flow, apart from a small fraction due to blood flow in the bone and fat of the finger. The duration of iontophoresis with adrenaline that was required to suppress the flow was surprisingly long, in view of the fact that the current per square centimetre of skin was about double that used to suppress cutaneous blood flow in the forearm within 10–20 min by Cooper, Edholm & Mottram (1955) and Edholm, Fox & MacPherson (1956), who used the technique to permit estimates of muscle blood flow by venous occlusion plethysmography. The former authors, aware that earlier experiments of this kind might have given misleading results because of incomplete suppression of skin blood flow, obtained evidence that their procedure did cause adequate suppression in one skin area of one subject. However, their estimates of the effect of indirect heating on muscle blood flow differ from those made with a calorimeter-sonde by Barcroft, Bock, Hensel & Kitchin (1955). The long time needed to suppress skin blood flow by iontophoresis in the present experiments is particularly striking, as the solution used in them contained higher concentrations of adrenaline, 0·04 g/ml. as opposed to 0·0005 g/ml. used by Cooper *et al.* (1955) and Edholm *et al.* (1956), and virtually no other cations; the solutions used for the forearms contained in addition a quantity of sodium. The long and variable time required to suppress finger-blood flow is probably due to the large and uneven thickness of the epidermis of the finger, and to variations of this from subject to subject. Variations of this kind must exist to some extent in any skin area, and must limit the usefulness of adrenaline iontophoresis as a means of suppressing skin blood flow and permitting estimates of muscle blood flow even in warm limbs. Clarke, Hellon & Lind (1958) reported that iontophoresis with adrenaline did not prevent cold vasodilatation in the forearm, and concluded that the

dilatation probably took place mainly in muscle blood vessels. However, they pointed out that the technique might not be reliable at these temperatures, and the present experiments show that adrenaline iontophoresis cannot be relied on to suppress skin blood flow at low temperatures.

SUMMARY

1. Adrenaline or noradrenaline were introduced by iontophoresis into the index fingers of men and women in a hot room, using a current of 2 mA.

2. At least 45 min of iontophoresis with either drug was required to reduce the blood flow to a negligible level in men, but in women a shorter time was often sufficient.

3. When fingers exposed to iontophoresis were immersed in ice-water a high blood flow appeared in them after a few minutes. The flow then became stabilized at a rather lower level.

4. These results support the view that a major cause of cold vasodilatation is impairment or loss of vascular response to constrictor hormones at near freezing-point.

5. They also show that iontophoresis with adrenaline is not a reliable method for distinguishing skin from muscle blood flow in cold limbs.

I am indebted to members of the staff of the laboratory who acted as subjects.

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CONTRACTURES AND PERMEABILITY CHANGES PRODUCED BY ACETYLCHOLINE IN DEPOLARIZED DENERVATED MUSCLE

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Acetylcholine (ACh) depolarizes the end-plate region of skeletal muscle by increasing its permeability to inorganic ions (Fatt & Katz, 1951). The application of radio-isotopes to the study of this action is technically difficult because in normal muscle ACh is effective at only a small fraction of the total membrane. After denervation, however, the sensitive area spreads to involve the entire fibre surface (Axelsson & Thesleff, 1959; Miledi, 1959, 1960), and large effects of ACh on the rate of loss of labelled potassium ions from the whole muscle can then be demonstrated (Klaus, Kuschinsky, Lüllmann & Muscholl, 1959).

In the present experiments radio-isotopes of sodium, potassium, calcium and chloride have been used to examine the effect of ACh on ion flux in the denervated rat diaphragm. In all experiments the muscles were equilibrated in solutions containing sufficient potassium to depolarize the fibres. Under these conditions ACh was still effective in accelerating ion movement (cf. Klaus *et al.* 1959), which could then be studied uncomplicated by secondary changes due to alterations in membrane potential.

It was observed in the course of such experiments that the depolarized muscles still contracted in response to ACh, provided that the bathing fluid contained calcium. A similar finding has been reported for smooth muscle (Evans, Schild & Thesleff, 1958; Robertson, 1960; Edman & Schild, 1961; Durbin & Jenkinson, 1961*a*, *b*). Since much evidence suggests that calcium ions play an important part in the linkage between excitation and contraction in muscles with normal resting potentials (Lüttgau & Niedergerke, 1958; Niedergerke, 1959; Bianchi & Shanes, 1959; Frank, 1960), it was of special interest to study the calcium dependence of these contractures, and of the effect of ACh on permeability.

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METHODS

Rats were anaesthetized with ether, and the left hemi-diaphragm denervated by evulsion of the phrenic nerve in the neck. Muscles were used 7–24 days later and were tested for the absence of innervation by application of shocks to the remainder of the nerve trunk; if any part of the hemi-diaphragm contracted the preparation was discarded. Further evidence of denervation was obtained by noting the presence of fibrillation, and by observing the development of tension on applying ACh in the bathing fluid; normal muscle does not respond under these conditions.

It was important for tracer measurements that the muscles should have been largely free from other tissue. The ribs, and as much connective tissue as possible, were therefore detached with a sharpened needle, under a dissecting microscope. Only the anterior third of the diaphragm could be used for this purpose. Generally, 3–4 strips of muscle, together weighing 80–200 mg, were obtained from a single rat. The preparations were attached to frames made of fine glass tubing through which an appropriate gas mixture was passed, oxygenating and stirring the bathing fluid.

Solutions. During dissection, and in some control experiments, the preparations were bathed in a modified Krebs's fluid which had the following composition (mM): Na 150, K 5·0, Ca 2·0, Mg 1·0, Cl 148, H_2PO_4 1·0, HCO_3 12, glucose 12, and was aerated with 95 % O_2, 5 % CO_2 gas mixture (Liley, 1956).

To depolarize the muscle fibres, the strips were transferred to a solution containing (mM): K 142, Na 5·0, Ca 5·0, CH_3SO_4 134, SO_4 3·25, Cl 15, HCO_3 1·5, glucose 12. This was aerated with O_2, and is referred to hereafter as 'potassium solution'.

Potassium methylsulphate was obtained from Hopkin & Williams Ltd, and was of only moderate chemical purity. The main contaminants were K_2SO_4 (2·7 %) and a volatile substance which could be detected by its odour, and which was removed by prolonged exposure of the salt to a partial vacuum. Muscles bathed in potassium solutions made up from potassium methylsulphate which had been treated in this way contracted in response to ACh for as long as 12 hr. It should be noted that because of the presence of 3·25 mM sulphate, approximately 25 % of the calcium in the final solution was in a combined form.

Neostigmine methylsulphate (Prostigmin) 2×10^{-7} g/ml. was included in all bathing fluids.

Procedure with tracers

The preparations were first equilibrated in the potassium solution for 2 or more hours. In efflux experiments, tracer was included during at least 1 hr of this period, care being taken to ensure that active and inactive solutions were of the same chemical composition. After loading, the strips were drained of adhering droplets and were then passed through a series of known volumes (1·2–5·0 ml.) of inactive potassium solution, to some of which ACh had been added. At the end of the experiment the strips were dissolved either in concentrated nitric acid or in warm 0·2 N-sodium hydroxide (cf. Durbin & Jenkinson, 1961*a*). Samples of the wash fluids and of the dissolved muscles were taken for counting.

Measurements of the effect of ACh on the influx of tracer were carried out on pairs of bone-free strips from single hemi-diaphragms. After equilibration for at least 2 hr, both strips were placed for 3 min in radioactive solution. This was to ensure that the interspaces should have contained tracer at the time of application of ACh. After this 'pre-load' period, the preparations were transferred for 5 min to separate samples of the same load solution, to one of which ACh had been added. The strips were then washed in known volumes of inactive fluid, as in efflux experiments. Finally they were weighed and dissolved for counting. By determining the activities of the wash fluids and of the muscles, the amounts of tracer remaining in the tissues at various times after removal from load could be calculated and compared.

A slightly different procedure was used in some early uptake experiments in which muscle strips with the bone still attached were used. After loading and washing for a standard period as before, the strips were blotted lightly and small portions dissected from the centre of each. These were rapidly weighed and then dissolved for counting; the wash fluids were discarded. Although this method was less time-consuming, the use of bone-free strips was preferred as the additional information gained allowed the rates of loss of tracer from the preparations to be compared.

Measurement of radioactivity. One-millilitre samples of the wash fluids were dried on planchettes under a lamp and counted with an end window G–M tube. To determine the activities of dissolved muscles containing either ^{42}K or ^{24}Na, the solutions were made up to 10 ml. with the standard potassium fluid, and 1 ml. samples taken for counting; the weights of material on the 'muscle' and on the 'wash' planchettes were then almost identical. However, this was not so in experiments with ^{45}Ca or ^{36}Cl when it was necessary, in order to obtain a satisfactory count rate, to transfer all the dissolved muscle to the final planchette. An allowance for the increased self-absorption of this sample had therefore to be made. The correction to be applied was determined by measuring the reduction in count rate produced by the addition of known amounts of either potassium methylsulphate or sucrose to an identical planchette containing a sample of the appropriate isotope. In the most extreme case, when the weight of material (containing ^{45}Ca) on the final planchette exceeded that on the others by 3·7 mg/cm^2, the correction amounted to 20 %.

The count rates were also adjusted to allow for background, decay, and if nickel and platinum planchettes were used in the same experiment, for differences in back-scattering. When assaying either ^{36}Cl or ^{45}Ca, a disk of lens paper was placed in the planchette to promote even spreading of the salts on drying. Nevertheless, all samples containing ^{45}Ca were redissolved, dried and counted at least twice more to obtain consistent results.

Differences in the properties of the isotopes allowed the exchange of two ions to be followed simultaneously by the method described in a previous paper (Durbin & Jenkinson, 1961*a*). All samples of ^{42}K were found to be free of the long-lived contaminant which had occasionally complicated the application of the technique in the earlier investigation.

Recording

Mechanical recording. Strips of diaphragm with the bone still attached were mounted under isometric conditions in a water-jacketed bath. The lower end of the preparation was attached to a glass hook, and the upper, through a short length of stainless-steel wire, to the anode of a mechano-electric transducer valve (RCA 5734). The output, after amplification, was fed to a pen recorder.

Electrical recording. Membrane potentials were measured with intracellular micro-electrodes having a tip resistance of more than 10 MΩ. In some experiments, the effect of ACh was examined in the way described by del Castillo & Katz (1955). Two micro-electrodes were inserted into a fibre, one being used to pass current pulses of constant intensity, and the other to record the resulting changes in potential across the membrane. On briefly interrupting the 'braking' current preventing the diffusion of ACh from a nearby micro-pipette, the simultaneous effects of the drug on membrane potential and resistance could be followed.

Temperature. The experiments were performed at room temperature (18–23° C) with a few exceptions which are indicated in the text.

RESULTS

Effect of ACh on outward movement of sodium and potassium

Figure 1*a* illustrates the time course of loss of ^{42}K from a denervated diaphragm bathed in the standard potassium solution. The efflux occurred in two phases which corresponded, to a first approximation, to successive clearing of the interspaces and of the cells. Similar curves were obtained with ^{24}Na. Application of ACh during the second phase invariably resulted in a striking increase in the rate of loss of both ^{42}K and ^{24}Na (Figs. 1*b*, 2, 4 and 5). This effect was not maintained during the presence of ACh,

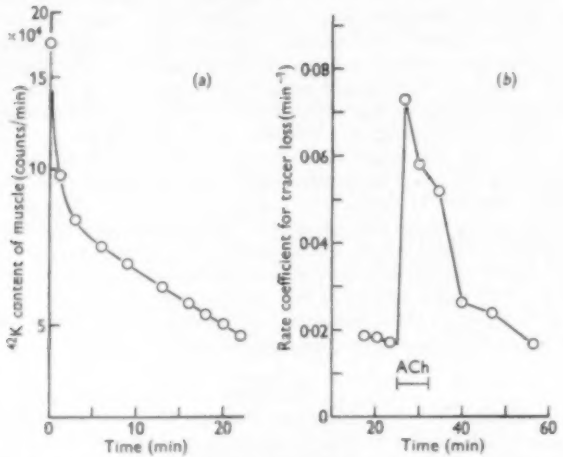

Fig. 1(*a*). Loss of ^{42}K from a bone-free strip of rat diaphragm (denervated 11 days previously) bathed at room temperature in the potassium solution used throughout the present study. Ordinate, amount of radioactivity remaining; abscissa, time from transfer of the muscle from solution containing ^{42}K. Semi-log. scale. (*b*). Effect of ACh ($7{\cdot}5 \times 10^{-6}$ g/ml.) on the efflux of ^{42}K from a strip of denervated muscle (16 days) from another rat. Ordinate, rate coefficient, defined as $\Delta c/\Delta t.c_m$, where Δc is the amount of radioactivity lost from the muscle during the collection period, Δt, and c_m is the mean of the tracer contents of the muscle at t and at $t+\Delta t$. Abscissa, as in (*a*). The points have been drawn at times corresponding to $t+\frac{1}{2}(\Delta t)$ in each period.

most probably because of the onset of 'desensitization' of the receptors (cf. Katz & Thesleff, 1957). It was also noted that the increase in rate coefficient (i.e. the proportion of muscle tracer lost in unit time) always persisted for some minutes after return of the strip to ACh-free solution. It is likely that this delay reflects the time required for ACh, and the tracer which exchanges as a result of the action of the drug, to diffuse from

the interspaces (cf. Krnjević & Mitchell, 1960). Because of these factors, and also since preparation of strips of diaphragm inevitably entailed some damage to the tissue, a quantitative analysis of the action of ACh was not attempted. However, to enable the effect on the exchange of different isotopes to be compared, ACh was usually applied at a concentration of $7{\cdot}5 \times 10^{-6}$ g/ml. for an arbitrary period of 7 min. The maximum increases in rate coefficient produced by this standard application were expressed as percentages of the rates in the absence of ACh, as estimated by interpolation. Values obtained in this way are listed in Table 1 (cols. 1 and 3).

TABLE 1. The effect of the standard application of ACh ($7{\cdot}5 \times 10^{-6}$ g/ml., for 7–8 min) on the exchange of different labelled ions. The percentage increases have been calculated, as described in the text, from the results of experiments of the type illustrated in Figs. 1*b* and 3. — indicates a negligible effect (< 5%).

1	2	3	4	5	6	7	8
^{42}K		^{24}Na		^{36}Cl		^{45}Ca	
Efflux	Uptake	Efflux	Uptake	Efflux	Uptake	Efflux	Uptake
105	110	150	310	—	10	—	280
330	40	100	150*	40	50	—	40
220	50*		410*	15	—	—	100
				20†	30*	—	380*
				7			120*
							220*

* These values were obtained with pieces of diaphragm with the bone attached (see Methods) and have been calculated from the amounts of tracer remaining in the muscles approximately 15 min after removal from load.

† Concentration of ACh increased to 2×10^{-5} g/ml.

In one experiment ACh was applied twice to the same strip, first at 10^{-7} g/ml. and then 45 min later at $7{\cdot}5 \times 10^{-6}$ g/ml., and was observed to increase the rate of loss of ^{42}K by 50 and 200% respectively.

As described more fully in a later section, ACh also caused the diaphragm to contract even though the fibres were depolarized. This response failed in the absence of calcium. It was therefore of interest to determine whether the effect of the drug on ion flux was also calcium-dependent. To test this point, two strips of a denervated muscle were loaded with ^{42}K in the standard potassium fluid. They were then transferred to separate inactive solutions, one of which was calcium-free. Twenty minutes later ACh was applied to each strip; the resulting increases in rate of loss of tracer are shown in Fig. 2. It is clear that this action of ACh was in fact little affected by calcium lack.

Effect of ACh on inward movement of sodium and potassium

Further evidence that ACh increases the permeability of the membrane to sodium and potassium ions was obtained by examining its effects on the

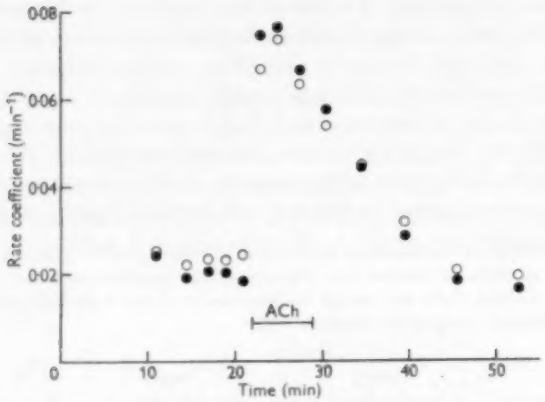

Fig. 2. Test of calcium-dependence of the effect of ACh on the efflux of ^{42}K. Two bone-free strips of a denervated diaphragm (11 days) were exposed to the same concentration ($7{\cdot}5 \times 10^{-6}$ g/ml.) of ACh in separate potassium solutions, only one of which contained calcium. The resulting increases in rate are seen to be about the same (○, Ca 5 mM; ●, Ca-free). Plotted as in Fig. 1*b*.

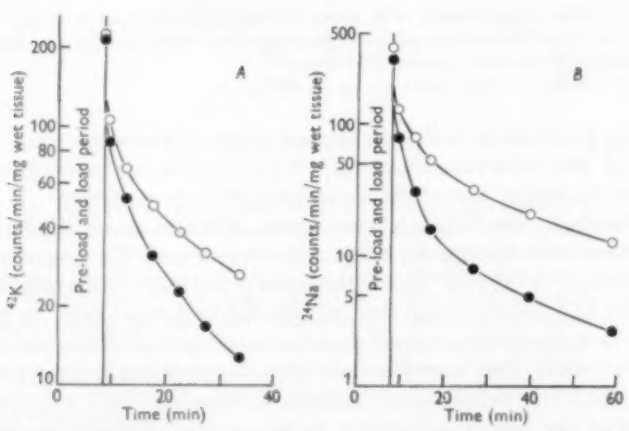

Fig. 3. Effect of ACh on the uptake of ^{42}K (*A*) and ^{24}Na (*B*). In each experiment, two bone-free strips of a denervated diaphragm (*A*, 7 days; *B*, 18 days) were exposed, after a brief 'pre-load' period, to separate radioactive solutions, to one of which ACh ($7{\cdot}5 \times 10^{-6}$ g/ml.) had been added. The strips were then returned to inactive fluid, and the loss of tracer observed; the amounts remaining in each (○, ACh-treated; ●, control) have been plotted as in Fig. 1*a*. Semi-log. scales.

uptake of ^{42}K and ^{24}Na. Two muscle strips were exposed, as described in Methods, to separate load solutions, one of which contained ACh. The subsequent procedure is illustrated in Fig. 3 (cf. Fig. 5). It can be seen that immediately after removal from load solution the ratio of the tracer contents of the 'ACh' and 'control' strips was close to unity, most probably because the activity in the interspaces of both formed a substantial fraction of the total uptakes. However, when the loss of tracer to inactive solution had continued for some minutes the efflux curves flattened and began to diverge, suggesting that the extracellular component had become sufficiently reduced for differences in a slower fraction, which presumably corresponded to exchange of tracer in the cells, to become apparent. In every experiment the ACh-treated strip was found to contain more activity, from which it is reasonable to conclude that the preceding uptake had been accelerated. Had thinner preparations been available it might have been possible to distinguish between tracer in the cells and in the interspaces by extrapolating the later part of the efflux curves to the time of transfer of the strips from load (cf. Durbin & Jenkinson, 1961*a*). However, in the present experiments this extrapolation could not be made with confidence because the proportion of extracellular tracer was high, and its loss relatively slow. Instead, an approximate measure of the action of ACh on uptake was obtained by expressing the difference between the tracer contents of the 'ACh' and the 'control' strips after an arbitrary period of 25 min in inactive solution (by which time the extracellular component will have been reduced to a small fraction of its original value) as a percentage of that remaining in the control. Values obtained in this way have been included in Table 1 (cols. 2 and 4). It may be noted that they cannot be compared directly to those which express the effect on efflux, as the experimental procedures differed in several respects. Nevertheless, it can be concluded from these results that ACh accelerated both inward and outward movement of sodium and potassium.

ACh and exchange of chloride

The rate of exchange of chloride was little affected by ACh. This was most clearly shown by double tracer experiments in which the actions of ACh on the efflux of ^{36}Cl and either ^{24}Na or ^{42}K (e.g. Fig. 4*A* and *B*) were determined with the same preparation.

As can be seen in Fig. 4, the increase in the rate of loss of chloride barely exceeded the scatter of the experimental points; although estimates of the percentage change in rate coefficient have been given in Table 1 (col. 5), the values are to be regarded as upper limits. It was concluded that the effect of the standard application of ACh on the loss of chloride was at most 15% of that observed with sodium and potassium.

Four experiments were made to test the action of ACh on the uptake of labelled chloride; the results are listed in Table 1, col. 6. As noted in Methods, uptake measurements required the preparation of two bone-free strips which may have differed in the relative proportions of damaged tissue. The results were therefore subject to considerable inaccuracy and can only be taken to suggest that the effect of ACh on uptake, as on efflux, of chloride is relatively small.

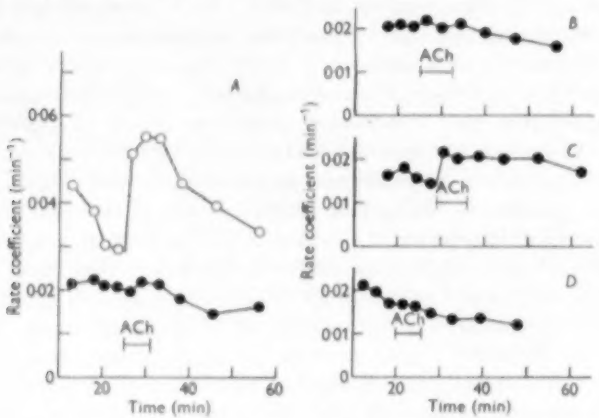

Fig. 4. Effect of ACh ($7{\cdot}5 \times 10^{-6}$ g/ml.) on the efflux of ^{36}Cl (●), plotted as in Fig. 1*b*. *A* and *B* are from 'double tracer' experiments, the second isotope in *A* being ^{24}Na (○). In *B*, ^{42}K and ^{36}Cl were combined; the increase in potassium flux is illustrated separately (Fig. 1*b*). Days of denervation for *A*, *B*, *C*, *D* = 19, 16, 24 and 11 respectively.

ACh and exchange of calcium

An experiment to test the effect of ACh on both uptake and efflux of ^{45}Ca and ^{24}Na is illustrated in Fig. 5. It will be seen from *A* that the strip loaded in the presence of ACh contained a greater amount of ^{45}Ca. This was a consistent finding, the magnitude of the effect being of the same order as that observed with sodium and potassium (cf. Table 1, cols. 2, 4 and 8). It seems reasonable to conclude, as before, that the divergence of the curves is a consequence of the effect of ACh in accelerating the preceding uptake. However, no corresponding increase in the rate of loss of labelled calcium could be detected. The possibility that this might have been due to some deterioration of the preparation was excluded by double-tracer experiments (e.g. Fig. 5(*B*)), in which ACh was found to accelerate the loss of either ^{24}Na or ^{42}K in the usual way.

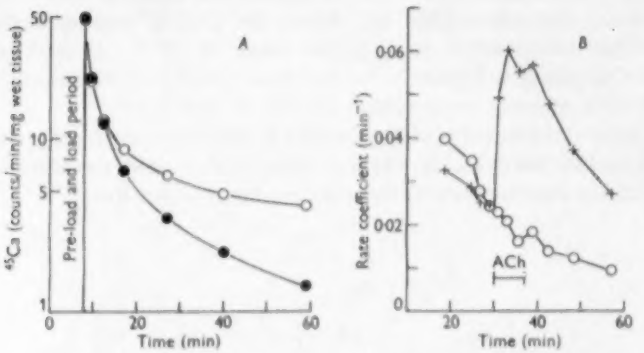

Fig. 5. 'Double tracer' experiment using three strips from the same diaphragm (18 days) to test the effect of ACh ($7{\cdot}5 \times 10^{-6}$ g/ml.) on the uptake (*A*) and efflux (*B*) of ^{45}Ca and ^{24}Na. *A* (plotted as in Fig. 3; semi-log. scale) shows only the curves obtained with ^{45}Ca (○, ACh-treated strip; ●, control); the corresponding results for ^{24}Na are illustrated in Fig. 3(*B*). In *B* (plotted as in Fig. 1*b*), the rates of loss of ^{24}Na and ^{45}Ca are indicated by the symbols + and ○, respectively.

ACh contractures of depolarized muscles

At room temperature the denervated rat diaphragm responded to immersion in the standard potassium solution with a transient contracture. In four experiments the tension reached its peak in from 0·5 to 2·5 min, and thereafter returned to the previous value at rest with a half-time of 1–6 min.

When the 'potassium contracture' had subsided, the application of ACh still caused the muscle to develop tension (Fig. 6). The time course of this response varied considerably from preparation to preparation. Small doses of ACh elicited a slow contracture requiring 2–5 min for its completion (cf. Fig. 6*D*). With maximal or near-maximal doses, however, the tension reached its peak in from 7 to 50 sec (mean value in 11 experiments, 15 sec), and had fallen to half 15–70 sec (mean value 40 sec) later. This variability, which could not be attributed to differences in the period of denervation, provided one of the most puzzling features of the present work.

The contractures were reversibly abolished by tubocurarine (3×10^{-6} g/ml.). In other respects, however, the response to ACh changed on depolarizing the muscle. In the first place, greater concentrations of ACh (10–30 times as much, for just detectable contractures) were required. Also, the maximum tension was never more than 40%, and sometimes less than 10%, of that in Krebs's fluid (cf. Fig. 7).

Further, the ACh contractures of depolarized muscles were markedly temperature-dependent (Fig. 8). Above 30° C ACh became ineffective, the optimal temperature being in the range 18–22° C. In contrast, denervated diaphragms bathed in Krebs's fluid contract strongly on exposure to ACh both at room temperature (cf. Fig. 6) and at 37° C.

Below 15° C the response of depolarized muscles to successive applications of ACh rapidly declined. It was also noted that at such temperatures the preparations showed apparently spontaneous contractures.

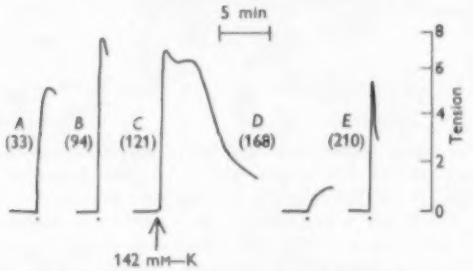

Fig. 6. Contractures of a denervated hemi-diaphragm (13 days), recorded under isometric conditions at 18° C. *A* and *B* show the response to ACh (10^{-6} and 10^{-5} g/ml. respectively, added at dots) in Krebs's fluid. At *C*, the standard potassium solution was introduced. When the resulting contracture had subsided, ACh was still effective (*D*, 10^{-6}; *E*, $2{\cdot}7 \times 10^{-5}$ g/ml.). '8' on tension scale corresponds to 8 g for *A*, *B* and *C*, and to 3·2 g for *D* and *E*. Passive tension due to stretch of muscle was less than 0·3 g throughout. The time (min) from mounting of the strip is given with each record.

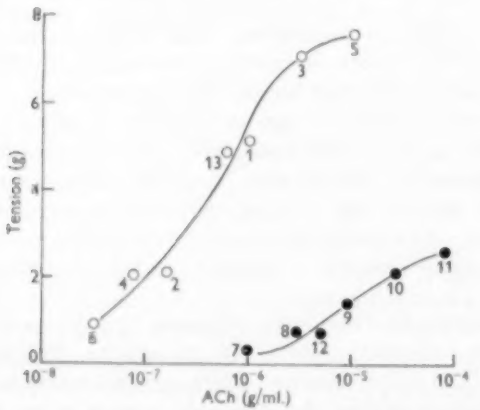

Fig. 7. Peak tension developed in response to ACh applied in Krebs's (○) and in potassium (●) solutions. From same experiment as in Fig. 6. The numbers indicate the order in which the measurements were made. Semi-log. scale.

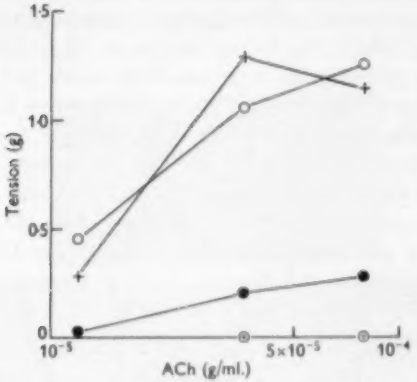

Fig. 8. Mechanical response of a denervated diaphragm (14 days) to ACh applied in potassium solution at 18 (+), 21 (○), 27 (●), and 37 (⊙) °C. On reducing the temperature to 15° C, the effectiveness of ACh fell rapidly, becoming negligible after 30 min. Semi-log. scale.

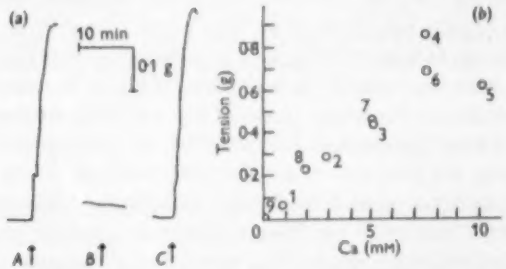

Fig. 9(*a*). Response of a depolarized diaphragm (denervated 18 days) to ACh (10^{-4} g/ml.) applied at intervals of approximately 13 min. Calcium was withdrawn immediately after *A*, and restored after *B*. (*b*). Peak tension developed by another preparation (22 days) in response to ACh (10^{-5} g/ml.) applied in potassium solutions containing different concentrations of calcium. The numbers indicate the order in which the measurements were made.

Calcium dependence of the contractures

The ACh contractures of depolarized diaphragms did not require the presence of either sodium or chloride ions in the bathing fluid and could be observed in preparations soaked at room temperature in isotonic potassium sulphate, saturated with calcium sulphate, for as long as 6 hr. The response was, however, graded with the external calcium concentration, and became negligible 5–15 min after withdrawal of this ion (Fig. 9). On restoration of calcium, recovery took place within 5 min. In contrast,

a similar period of calcium lack had little or no effect on the ACh contractures of diaphragms bathed in Krebs's fluid, other experimental conditions, e.g. temperature (18–22° C), being the same. This difference could be clearly shown by transferring a preparation which had been soaked for 1 hr in calcium-free potassium solution to calcium-free Krebs's fluid; after 3 min ACh, which had previously been quite ineffective, caused the muscle to contract strongly. It should, however, be noted that no rigorous precautions were taken to eliminate traces of calcium, so the possibility remains that the ACh contracture in Krebs's fluid may also fail in the complete absence of calcium ions. The amounts of ionized calcium in 'calcium-free' potassium and Krebs's fluids are, however, most probably of the same order. It therefore seems likely that there is a considerable quantitative difference in the calcium dependence of the responses to ACh in these fluids.

The effect of ACh on the membrane potential of denervated muscles in the standard potassium solution

It was important to know whether the actions of ACh described in the preceding sections were associated with potential changes. Micro-electrodes were used to obtain information on this point. In one experiment, the effect of applying ACh (2×10^{-5} g/ml.) to a denervated diaphragm bathed in potassium fluid was tested. It was found difficult to make satisfactory measurements during the rising phase of the resulting contracture. However, when the peak tension had been reached, the mean potential observed in eleven fibres, $2{\cdot}6 \pm 0{\cdot}7$ mV (S.E. of mean), did not differ significantly from the pre-ACh value of $3{\cdot}0 \pm 0{\cdot}3$ mV (20 fibres). Although this suggested that ACh had produced little change, the method was abandoned because of the difficulty of making rapid measurements of membrane potentials lying within a few millivolts of zero in a mechanically active muscle.

More conclusive evidence was obtained by examining the simultaneous effects of highly localized applications of ACh on the potential and resistance of denervated muscle fibres. Two micro-electrodes were inserted into a fibre, one being used to record the changes in membrane potential caused by the passage of current pulses through the other (Fig. 10*A*). When ACh was released from an extracellular micro-pipette, the electrotonic potential became smaller and more rapid, indicating a fall in membrane resistance. There was, however, little shift of the base line. This is illustrated more clearly in Fig. 10*B*, which shows the effect of ACh on a series of electrotonic potentials. Similar results were obtained from nine fibres in two denervated muscles; although small changes of potential in either direction were sometimes observed, their magnitude never ex-

ceeded 2 mV. Thus it could be concluded that concentrations of ACh sufficient to produce a marked increase in membrane conductance caused little change in potential.

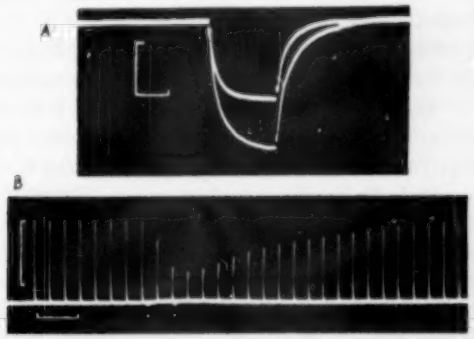

Fig. 10. Effect of ACh on electrotonic potentials recorded from a single fibre in a denervated diaphragm (10 days) bathed in the standard potassium fluid. *A*: 'Repolarizing' potentials superimposed. Between the sweeps ACh was applied from a nearby micro-pipette. Scales: 5 mV and 10 msec. *B*: 'Depolarizing' potentials recorded on slowly moving film. Between dots, the 'braking' current through the micro-pipette was interrupted so as to release ACh. Scales: 5 mV and 1 sec.

DISCUSSION

Effect of ACh on tracer movement

Under the conditions of the present experiments ACh had little if any effect on the membrane potential, which lay within a few millivolts of zero. For this reason, and because net movements of those ions which readily penetrate the membrane were probably largely complete by the time of application of ACh, the observed increases in the rate of exchange of ^{24}Na and ^{42}K must be attributed to an enhanced permeability to sodium and potassium.

It may be noted that no comparable effect can be detected in the innervated diaphragm (Klaus *et al.* 1959), presumably because there are then relatively few receptors so that changes in ion flux resulting from the action of ACh are masked by the exchange of tracer across other parts of the membrane.

In contrast to the findings with sodium and potassium, ACh markedly increased the uptake of ^{45}Ca by the denervated diaphragm without having any corresponding effect on the rate of loss. It is, however, known that much of the calcium in the cells of frog skeletal muscle exists in a bound form (Gilbert & Fenn, 1957; Harris, 1957) and it seems reasonable to

assume that the same applies to the diaphragm. The rate of loss of tracer from a loaded preparation may therefore be determined largely by the kinetics of calcium movement within the cell. Failure to observe a direct effect of ACh on calcium efflux, despite an increased membrane permeability, would not then be unexpected.

The results obtained with ^{36}Cl show that a change in chloride permeability contributed relatively little to the increase in membrane conductance caused by ACh. It does not, of course, necessarily follow that the same holds for the action of the drug on either normal or denervated diaphragms bathed in more physiological solutions, and this remains to be investigated. Recently Takeuchi & Takeuchi (1960) have applied the 'voltage clamp' technique to the study of the effect of ACh at the end-plate region of innervated frog skeletal muscle. Their measurements indicated that the change in permeability extended to sodium and potassium, but probably not to chloride, being similar in this respect to that observed with the denervated rat diaphragm. In contrast, experiments on the exchange of labelled potassium, chloride and bromide in depolarized smooth muscle (the taenia coli of the guinea-pig) have shown that carbachol increases the permeability to all three ions to approximately the same extent (Durbin & Jenkinson, 1961*a*).

ACh contractures of depolarized diaphragms

The mechanical response of the denervated rat diaphragm to ACh applied in potassium-rich solutions resembled that of smooth muscle (Evans *et al.* 1958; Durbin & Jenkinson, 1961*a*, *b*) in several respects. In particular, the contracture could be abolished by an appropriate antagonist, e.g. tubocurarine in the case of the diaphragm, and by withdrawal of calcium. This is discussed below. Among the differences noted was that the diaphragm became much less sensitive to ACh on depolarization, whereas with some varieties of smooth muscle the reverse has been shown to occur.

With maximal or near-maximal doses of ACh, the response of the depolarized diaphragm became quite rapid, the average time required for the development of the peak tension being 15 sec. During this interval ACh cannot have diffused much beyond the superficial fibres, in which the contracture must have originated. For this reason little significance can be attached to the absolute values of the tensions developed under the conditions of these experiments.

Generally the tension declined almost as rapidly as it had risen, even though the bathing fluid still contained ACh. This may have been due to the onset of desensitization which was already apparent in tracer experiments in which ACh was applied at $7{\cdot}5 \times 10^{-6}$ g/ml. (Fig. 1*b*). As

more than ten times this concentration was normally required to elicit a maximal contracture, desensitization would have been expected to be rapid. A second possibility is that the muscle may have begun to relax even though the processes which activate contraction were still operative, i.e. that some form of 'de-activation' of the contractile mechanism occurred. Although the techniques used in the present work did not allow the relative importance of these factors to be determined, it is possible that several puzzling features of the contractures may prove explicable in such terms. For example, the failure of the response above 30° C may have been a consequence of either or both processes becoming more rapid at higher temperatures.

It was of special interest that the mechanical response of the depolarized diaphragm to ACh was dependent on calcium, for this ion is known to play an important part in the initiation of contraction in muscles under more physiological conditions. This has been studied in most detail in the heart where it has been shown (Lüttgau & Niedergerke, 1958; Niedergerke, 1959) that many features of the action of calcium can be explained by supposing that depolarization of the membrane, whether by the action potential or by the application of a potassium-rich solution, causes a negatively-charged calcium compound to move from a superficial to a deeper part of the cell, where it activates the contractile mechanism. There is some evidence to suggest that a somewhat similar process operates in skeletal muscle, although it is already clear that there are quantitative differences, e.g. in the sensitivity to a reduction in the external calcium concentration (Frank, 1960).

In discussing possible mechanisms for the ACh contracture of depolarized diaphragms it is first necessary to consider whether this response was also a consequence of an alteration in membrane potential. Although the failure to detect any consistent effect of ACh on potential suggested that this was not the case, it must be pointed out that it proved impossible to eject sufficient ACh from a micro-pipette to produce a visible contracture. Thus the possibility that larger amounts would have altered the potential cannot be excluded, although it seems unlikely.

A more plausible hypothesis is to suppose that the ACh contracture is a consequence of a calcium influx which results not from a change in potential but from the increased permeability of the membrane, as suggested on the basis of similar, but less complete, experiments with depolarized smooth muscle by Durbin & Jenkinson (1961*b*). The size of the response would then be expected to vary with the quantity of calcium entering, which would presumably depend in turn on (*a*) the concentration gradient for ionized calcium, and (*b*) the extent of the increase in permeability. The failure of the contracture in calcium-free solution is

probably a direct consequence of the decrease in (*a*), as the effect of ACh on the permeability to potassium, and presumably other ions, did not appear to be calcium dependent. On the other hand, the abolition of the response by tubocurarine can reasonably be attributed to a reduction in (*b*).

SUMMARY

1. Radio-isotopes were used to study the effect of ACh on the permeability of the denervated rat diaphragm to inorganic ions. The preparations were bathed at 18–23° C in solutions containing a high concentration of potassium (142 mM), in order to depolarize the fibres and so avoid secondary ionic movements associated with changes in membrane potential.

2. ACh markedly increased the inward movement of calcium, and both inward and outward movements of sodium and potassium. Only small effects on chloride flux could be detected.

3. At room temperature ACh still caused the depolarized denervated muscles to develop tension (up to 40% of the maximum in Krebs's fluid). This response was reversibly abolished by tubocurarine.

4. The contractures failed in the absence of calcium, although there was no corresponding reduction in the effect of ACh on membrane permeability.

5. Micro-electrodes were used to show that ACh produced little or no change in the membrane potential of diaphragms equilibrated in the potassium-rich solution.

6. It is suggested that the ACh contractures observed under these conditions are a consequence of entry of calcium into the cells, following the increase in membrane permeability.

We wish to thank Professor Sir Lindor Brown, F.R.S. and Professor B. Katz, F.R.S. for their advice and encouragement, and Mr J. L. Parkinson and Mr L. Ward for frequent assistance.

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DORSAL ROOT REFLEXES OF MUSCLE GROUP I AFFERENT FIBRES

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Investigations on the interaction between afferent fibres have largely been concerned with cutaneous afferent fibres (Barron & Matthews, 1935, 1938; Toennies, 1938, 1939), which are particularly involved both on the input and output sides of the dorsal root reflex (DRR). Toennies (1939) reported that tapping the patellar tendon gave a DRR into the saphenous nerve, but it probably was not due to muscle stretch receptors, because a similar DRR was produced by tapping the skin over the tibia. Brooks, Koizumi & Malcolm (1955) and Brooks & Koizumi (1956) showed for the first time that there was a DRR from muscle afferents to muscle afferents, but only when the spinal cord was cooled. There was also a DRR from muscle nerve to cutaneous nerve and vice versa. So far as these types of DRR were investigated, they resembled the cutaneous-to-cutaneous DRR.

Further investigation of the phenomena of central interaction between muscle afferent volleys has been undertaken because it has seemed likely that such interaction forms the basis of the depression which muscle afferent volleys exert on the monosynaptic excitatory post-synaptic potential (EPSP) produced in motoneurones by other muscle afferent volleys (Frank & Fuortes, 1957; Frank, 1959). A preliminary report of this investigation on dorsal root reflexes has already been published (Eccles, Kozak & Magni, 1960).

METHODS

Throughout the whole experiment, the animal (cat) was lightly anaesthetized by pentobarbital sodium. The spinal cord was exposed by a laminectomy from L2 to S1. It was severed at the upper L2 level, and the left L4–S2 ventral roots were divided. The various peripheral nerves employed in the investigation were dissected on the left side and mounted on platinum electrodes (interpolar distance in excess of 10 mm) that could be used either for stimulating or recording. Approximate monophasic conditions of recording from peripheral nerves were secured by the application of procaine to the killed ends of the nerves, but with many of our records there was still much diphasic distortion. Both the

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cord and leg regions were covered by warmed mineral oil contained in pools formed by skin flaps. The animal was fixed in the rigid frame and the intracellular recording from afferent fibres was performed as described by Eccles & Krnjević (1959*a*). The temperature of the animal was registered by a thermometer inserted through the back of the neck so that it rested just outside the thoracic cage. The temperatures of the oil baths were also recorded frequently. The temperature of the interior of the spinal cord would lie between the temperatures of the body and of the oil pool. Since these temperatures rarely differed by more than 2 or 3° C, no large error would be introduced by assuming that the body temperature was a measure of the cord temperature. When cooling the spinal cord the whole animal was cooled by cold applications and the heating of the oil pool was also turned down so that the temperatures of the oil pool and of the body were approximately the same.

The following nerves were prepared, and their identification symbols are also given: posterior biceps plus semitendinosus (PBST); anterior biceps plus semimembranosus (SMAB); medial gastrocnemius (MG); lateral gastrocnemius plus soleus (LGS); plantaris (PL); flexor digitorum longus plus flexor hallucis longus (FDHL); peroneus longus, brevis and tertius (P); deep peroneal to tibialis anticus and extensor digitorum longus, the branch supplying skin and the extensor digitorum brevis muscle being eliminated (DP); sural (S); the purely cutaneous component of superficial peroneal (SP). When MG and LGS are combined the symbol is (GS), while P + DP = (PDP).

RESULTS

General investigations on dorsal root reflexes (DRRs) evoked by muscle afferent volleys

In the cooled spinal cord of the cat maximum Group I afferent volleys in the posterior biceps-semitendinosus (PBST) nerve always produced large dorsal root reflex (DRR) discharges in the afferent fibres of muscle nerves (Figs. 1E, 4, 5, 6, 7). Group I volleys in that part of the peroneal nerve supplying the anterior tibial muscles (PDP, see Methods) were also effective in producing DRRs (Figs. 1G; 6). Synchronous volleys in PBST and PDP were particularly effective (Figs. 1C, F, I; 3A, I; 4F; 8C).

In confirmation of Brooks & Koizumi (1956), these DRRs from muscle afferent fibres to muscle afferent fibres were usually slight at normal body temperature (Fig. 1A–C), and increased progressively as the cord was cooled (Fig. 1D–F), being always very large at the low temperature of 30° C. In many preparations the DRRs were very large when the body temperature was no lower than 35–33° C (Figs. 3; 4F–J; 5B). A DRR has been observed when the body temperature was as high as 40·5° C (Fig. 1G–I).

In general these DRRs from muscle afferent to muscle afferent have closely resembled the DRRs generated by cutaneous afferent volleys in cutaneous afferent nerves (Toennies, 1938, 1939). The central latent period was never less than 4 msec, and central latencies as long as 10 msec were observed with weak DRRs at low temperatures (about 30° C). Large DRRs were often up to 20 msec in duration, and there were sometimes distinct initial waves at 3–6 msec apart (Figs. 2E–M; 5, 6, 7), which evidently

were produced by an approximate synchronization of repetitive discharges in many fibres. Such preferred latencies for the discharges have been reported by Frank (1953) and will be illustrated later with intracellular recording.

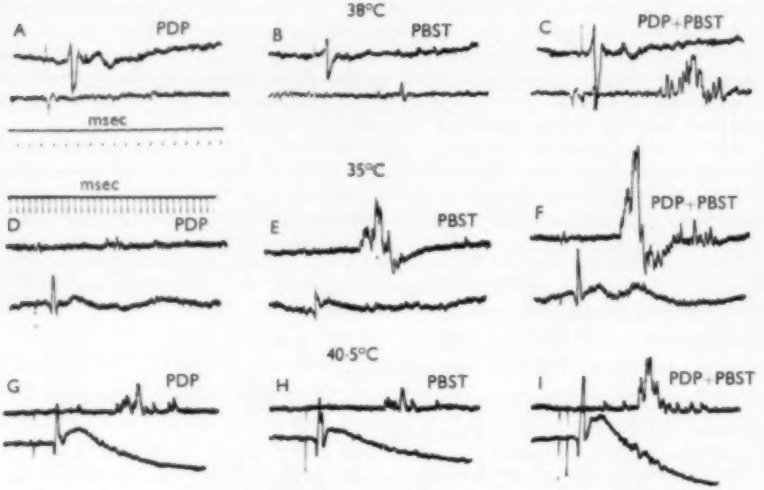

Fig. 1. Dorsal root reflexes (DRRs) produced by maximum Group I volleys in muscle nerves as indicated by the symbols above each record, and recorded in the FDHL nerve. In A–C the lower trace is from the FDHL nerve, the upper giving the potentials produced by the afferent volleys entering the cord through the L7 dorsal root, negativity being downwards. D–F were recorded later in the same experiment after the temperature had fallen from 38 to 35° C. The monitored record from the L7 dorsal root entry is now below the DRR record, and negativity is upwards. Note slower time scale for D–F. G–I are records as for D–F, but in another experiment with body temperature 40·5° C, and with time scale as for A–C.

When the DRR was small, a second volley at a brief interval (up to 5 msec) in the same afferent nerve caused a large increase in the DRR (Fig. 2A–D). With stimulus intervals longer than 10 msec there was depression of the DRR evoked by the testing volley, as was originally observed for cutaneous DRRs (Toennies, 1938). The depression gradually passed off as the testing interval was lengthened beyond some hundreds of milliseconds (Fig. 2E–I).

The prolonged depression that follows the production of each DRR by an afferent volley is well illustrated by observing the effects of repetitive stimulation. As is shown in the series of Fig. 2J–M, the DRR rapidly ceases, even during high-frequency tetanization. This complete suppression of the DRR is maintained so long as the tetanization is continued. Alternatively, the DRR can be observed at the steady state that is quickly

reached during sustained stimulation at relatively low frequency. As is shown in Fig. 3A–H, even a large DRR failed completely at a frequency of 5/sec, and almost no reflex survived at 2/sec. Over the frequency range 1/sec to 0·2/sec the DRR increased greatly, but only when the frequency was as slow as 0·14 to 0·1/sec was the DRR as large as with indefinitely long intervals between successive volleys. Figure 3I–L shows that even at a temperature as high as 40·5° C there is similar effect of suppression of the DRR as the frequency is raised to 0·2/sec and upwards. The small spike marked by an arrow is an example of a recurrent discharge, as discussed below. In the present investigation it has been a standard practice to evoke the DRRs at intervals of 7 sec, so as to avoid the depression.

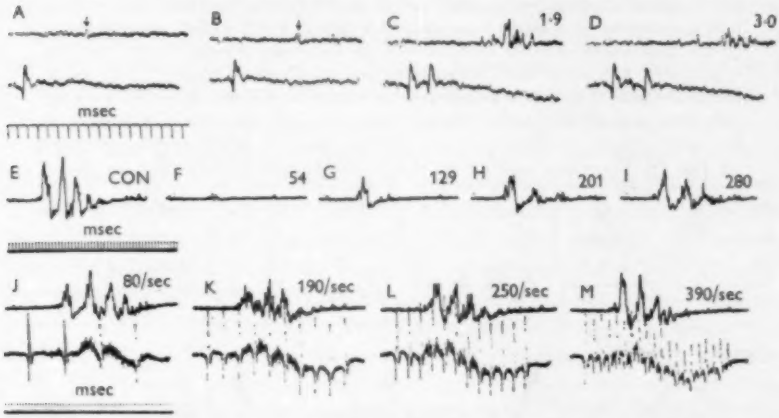

Fig. 2. Summation and depression of DRRs. A–D show DRRs (upper trace) and monitored dorsal root records produced by maximum Group I PBST volleys in MG nerve. Summed actions of two volleys at 1·9 and at 3·0 msec are seen in C and D, while A and B give control records for single volleys, there being DRRs in only one or two fibres. Body temperature 37° C. E shows control DRR discharged into the GS nerve in response to two maximum Group I PBST volleys at 3 msec interval. In F–I this double volley was employed as a test of the depression following conditioning by another similar double volley. The test intervals are indicated in msec. In J–M are DRRs produced in the GS nerve by repetitive Group I PBST volleys at the indicated frequencies, and as shown by the monitored records from the dorsal root in the lower traces. E–M are from the same preparation and at a body temperature of 31° C. Time scales are shown below each series.

Following a prolonged repetitive series of muscle afferent volleys there is rapid recovery from the depression (Figs. 2, 3) that occurs during the stimulation, and after several seconds a testing afferent volley usually evokes a larger DRR (Fig. 4B–D) than in the control responses before the tetanus (Fig. 4A). In every respect this potentiation is equivalent to the

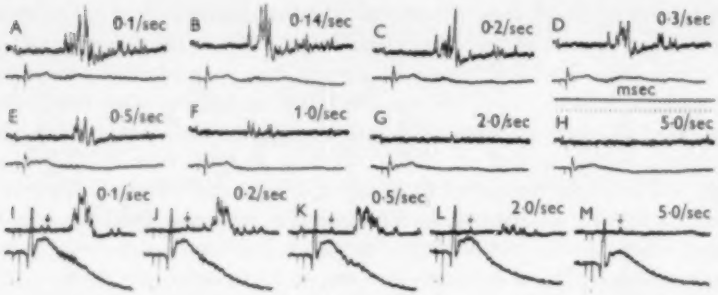

Fig. 3. Effect of frequency of stimulation on DRRs. A–H: Upper traces show DRRs produced by a single synchronized volley in PBST + PDP (maximum for Group I) and recorded in the FDHL nerve. The frequency of repetition is shown for each record, and below each record is the monitored record from L7 DR. Body temperature 33·5° C. I–M is a similar series in another preparation, but with a body temperature of 40·5° C. Arrows indicate a single impulse discharged with precisely the same latency at all frequencies. It is an example of a recurrent discharge (cf. Fig. 13). There is in addition an earlier spontaneous discharge in I and K.

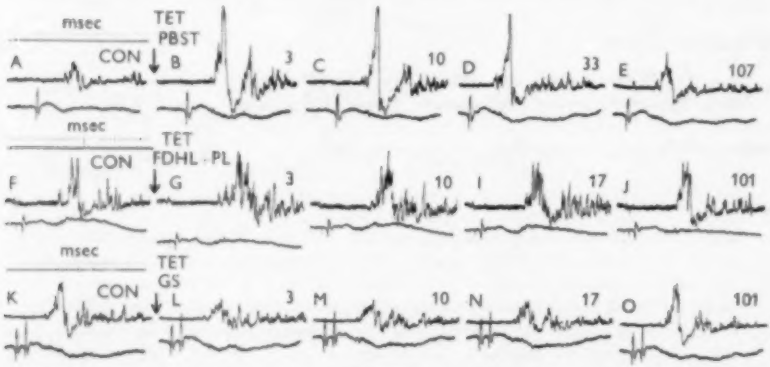

Fig. 4. Post-tetanic potentiation of DRRs. In series A–E a specimen control record is shown (A), evoked by a single PBST volley in GS nerve. Between A and B the standard conditioning tetanus of 300/sec for 15 sec was applied to the PBST nerve, and subsequently DRRs were evoked by testing PBST volleys at the times indicated in seconds after cessation of the conditioning tetanus. In F–J a synchronized PBST + PDP volley was employed to evoke a DRR into FDHL + PL nerve, F being control and G–J showing DRRs evoked at the indicated times in seconds after the end of a conditioning tetanus (300/sec for 15 sec) to the FDHL + PL nerve. K–O was from same preparation as A–E, but two PBST volleys evoked the DRR in GS nerve. The conditioning tetanus (300/sec for 15 sec) was applied as shown to the GS nerve and the times of the subsequent test volleys are shown in msec. Note time scales for each series. Body temperature for A–E and K–O was 31° C, for F–J, 33° C.

post-tetanic potentiation observed for conventional reflexes (Lloyd, 1949; Eccles & Rall, 1951; Wilson, 1955). After the standard conditioning tetanus of 300/sec for 15 sec, potentiation of the DRR persisted for at least 60 sec; no significant potentiation survived at 107 sec in Fig. 4E. The asynchronous nature of the discharge has prevented any accurate assessment of the degree of potentiation and hence no attempt has been made to plot the time course. The initial period of post-tetanic depression has been described for the DRRs evoked by cutaneous afferent volleys (Koketsu, 1956; Eccles & Krnjević, 1959*b*), but post-tetanic potentiation of cutaneous DRRs has not been reported.

When the DRR is large, post-tetanic potentiation is usually not observed with the DRRs from muscle afferents. In two experiments tetanization of a muscle nerve was not followed by potentiation of the DRRs evoked by another muscle nerve. However, further investigation is desirable before it can be concluded that DRRs never exhibit the cross-potentiation sometimes observed with the polysynaptic flexor reflex (Wilson 1955).

When the conditioning tetanus was applied to the muscle nerve in which the DRR was recorded, there was sometimes a fairly large post-tetanic potentiation of the DRR. As is illustrated in Fig. 4F–I, the potentiation was always largest immediately after cessation of the conditioning tetanus, even within 1 sec; all potentiation had disappeared in Fig. 4J at 101 sec. A more common post-tetanic effect has been a lengthening of the DRR, rather than a potentiation, which is seen by comparison between the control in Fig. 4F and the immediate post-tetanic records, G–I. Post-tetanic potentiation of the dorsal root potentials in cutaneous afferent fibres is a well attested phenomenon after tetanization of the recording afferent fibre (Woolsey & Larrabee, 1940; Lloyd, 1952; Eccles & Krnjević, 1959*a*), but no associated increase in the DRR has been described.

Types of muscle afferent fibres producing DRRs

Usually the PBST nerve exhibits an almost complete separation between the thresholds for the Ia and Ib afferent fibres respectively (Eccles, Eccles & Lundberg, 1957*a*; Laporte & Bessou, 1957). The best test of this separation is provided by the double-stimulation technique, where only those Group I afferent fibres not excited by the first stimulus can respond to a second stimulus that is just supramaximal for Group I and given about 1 msec later. Thus in Fig. 5A, B the first stimulus excited a large fraction (about two thirds) of the Group Ia fibres when at strengths of 1·38 and 1·64T respectively; yet, as shown in the corresponding records from the lateral gastrocnemius nerve, there was only a trace of DRR in both cases. The next strongest stimuli in Figs. 5A, B (1·70 and 1·85T respectively)

were maximal for the Group Ia fibres, but excited very few Ib fibres. There was correspondingly a large increase in the DRRs, which presumably was due to the considerable addition to the Ia volley, rather than to the few Ib impulses. However, the increase to a maximum Ib volley, with stimulus strengths of 2·0 to 2·46T in A and 2·06 ot 3·06T in B, further increased the DRR. Thus it can be concluded that in Fig. 5 the DRR was evoked by both the Ia and Ib afferent volleys. In Fig. 5A the large DRR at 1·70T indicated that the Ia volley was more powerful than the Ib, though the Ia volley had to be about two thirds maximal before it evoked any DRR. The relative potency of the PBST Ia volley in this same experiment is also illustrated by comparing the upper with the second row of records in Figs. 6 and 7. In other preparations (cf. Fig. 5B) the Ib volley appeared to be more powerful than the maximum Ia volley, the 3·06T response being compared with the 1·85T response. However,

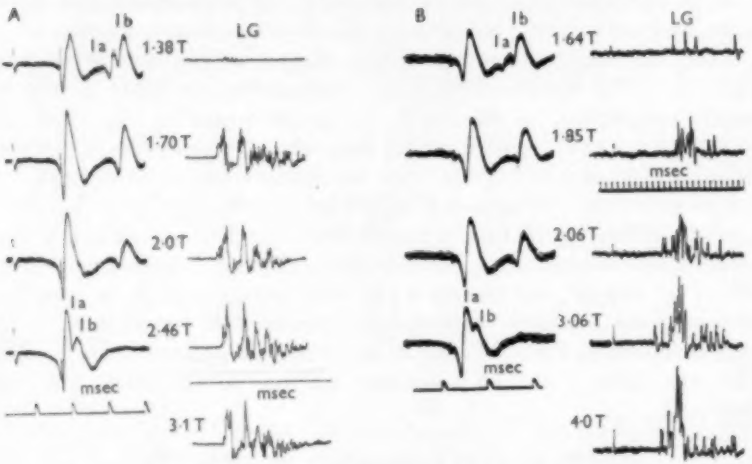

Fig. 5. Types of Group I impulses producing DRRs. In A single stimuli at the indicated strengths relative to threshold are applied to the PBST nerve and the dorsal root reflexes into LG nerve are in the right-hand column. In the left-hand column are, first, records produced by the PBST volley as it enters the cord through the L 7 and S 1 dorsal roots, and secondly, the responses evoked by a second stimulus applied 1·3 msec later through the same electrode on the PBST and at a strength just supramaximal for Group I. For that stimulus strength the interval is briefer than the refractory period, so it only excites fibres not excited by the first stimulus, which is thus shown to be maximal for Group I at 2·46T, and respectively submaximal and maximal for Group Ia at 1·38 and 1·70T. Time scales are shown for each column. Body temperature 31° C. B is a similar series from another experiment with body temperature 34° C. It is to be noted that in A and B the DRRs were evoked by the single stimuli that gave the first responses in the dorsal root records. These latter records were taken just subsequent to the DRR series.

in producing DRRs the requirement of summation is so dominant that the relative magnitudes of the Ia and Ib contributions cannot be derived from series such as those illustrated; nevertheless, they establish that both the Ia and Ib afferent impulses from muscle are concerned in the production of DRRs.

An invariable finding has been that increasing the stimulus beyond the maximal strength for Ib caused little or no further increase in the DRR, either in intensity or duration. For example, the stimulus strengths of 3·1T and 4·0T in Figs. 5A and B respectively would have excited many of the Group II afferent fibres, yet there was no appreciable increase in the DRRs. Evidently Group II and III afferent impulses from muscle are virtually ineffective in evoking DRRs in the large muscle afferent fibres.

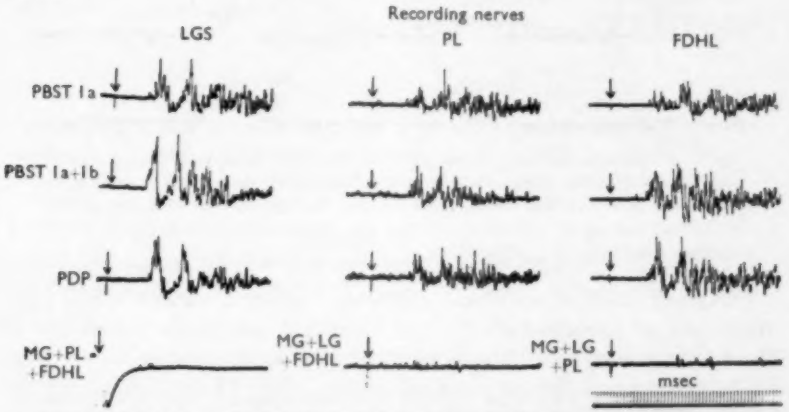

Fig. 6. DRRs into nerves to the post-tibial extensor muscles. The respective recording nerves are specified above the columns and volleys producing the DRRs are indicated to the left of the top three rows. In the lowermost row the volleys are different for each column, as shown, being applied in each case through the three nerves to the post-tibial extensor muscles that were not used for recording. Stimuli just maximal for Group I were applied to the PDP, MG, LG, PL and FDHL nerves. Body temperature 31° C.

Hitherto the general properties of the DRRs discharged into muscle afferent nerves have been illustrated with examples in which afferent volleys in the posterior biceps-semitendinosus (PBST) or peroneal (PDP) nerves evoked DRRs into the LGS, PL or FDHL nerves, as illustrated in the three upper rows of Fig. 6. Such DRRs are amongst the most readily evoked. When a systematic study was made of the DRRs discharged into many species of muscle nerves in response to many species of muscle afferent volleys, it was found that other muscle afferent volleys also could

produce DRRs, though they were always less effective than the PBST or PDP volleys. It was also found that any particular muscle afferent volley would produce DRRs in any muscle nerve with an approximately equivalent effectiveness, even into itself. For example, in Figs. 6 and 7 PBST volleys evoked large DRRs in LGS, PL, FDHL, P, and DP nerves. The smaller responses of the P nerve are at least in part attributable to the shunting by the superficial peroneal nerve which was left attached, but cut centrally.

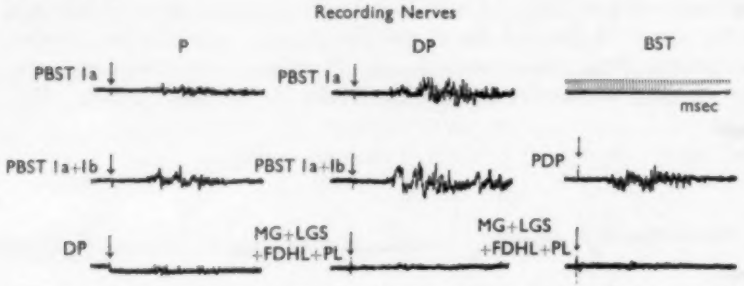

Fig. 7. DRRs into nerves to flexor muscles. The series are arranged as in Fig. 6, with recording nerves specified above each column, while the volleys producing the DRRs are specified just before each record. Except for the first two PBST volleys all volleys are evoked by stimuli just supramaximal for Group I. Body temperature 31° C.

The much lower effectiveness of Group I afferent volleys in the post-tibial extensor muscles (GS, PL and FDHL) is seen in the lowest row of Fig. 6, where there are barely detectable DRRs in contrast to the large DRRs produced in the same muscle nerves (LGS, PL or FDHL), by the PBST or PDP volleys. Similarly, in the lowest row of Fig. 7 a combined afferent volley from these three post-tibial extensor muscles produced an extremely small DRR into DP or BST nerves, which contrasted with the large DRRs produced by PBST or PDP volleys in the row above.

Since a considerable summation of excitatory actions is required in order to evoke a DRR, a more sensitive test for the effectiveness of an afferent volley would be to superimpose it upon a conditioning afferent volley that alone produced a DRR. For example, in the series of Fig. 8 a single PBST volley was employed for this purpose and superimposed thereon in C, G, K and O were single volleys in PDP, vastocrureus (VC), SMAB and GS nerves respectively. It is seen that each of these volleys increased the DRR above the PBST response in A, E, I, M, though with each alone (B, F, J, N) there was an appreciable DRR only to the PDP volley. It is further shown in D, H, L, P that brief repetitive stimulation (6 volleys at 280/sec) was much less effective in displaying the ability to produce

DRRs than was summation with the PBST volley. By this method of summation it has been shown that Group I afferent volleys from all tested hind-limb muscles share to some extent the ability to produce DRRs in the muscle nerves of that limb. It has further been found that this effectiveness was not appreciably diminished when there was segmental separation between the afferent volleys and the muscle nerve in which the DRR was recorded. For example, PBST volleys entering the cord at the L7–S1 level were very effective in evoking DRRs in the afferent fibres of vasto-crureus muscle, which have a segmental level of L5 to upper L6.

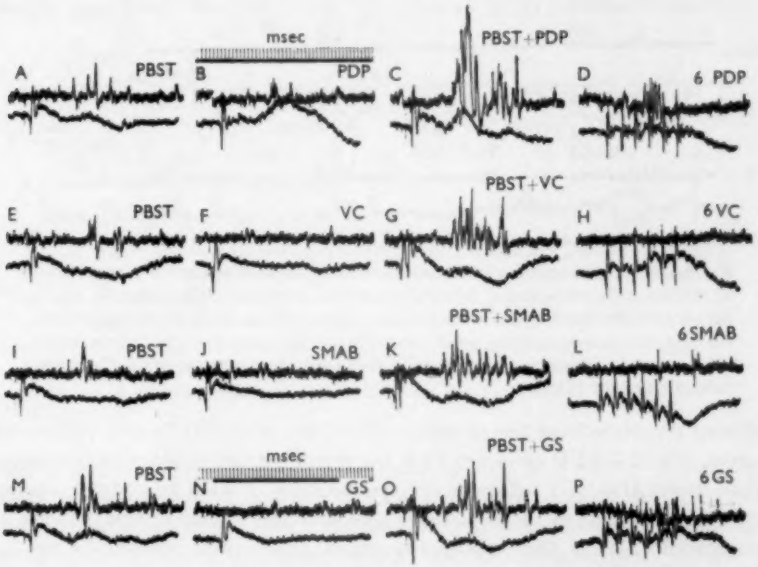

Fig. 8. DRR production by maximum Group I afferent volleys from different muscles. A, B and C show DRRs into the FDHL nerve produced by single PBST, PDP and combined PBST and PDP volleys respectively. There is a large DRR in C and in D a much smaller DRR is produced by 6 PDP volleys at 280/sec. Note the dorsal root records immediately below the DRR records. E–H, I–L and M–P are similar series for the VC, SMAB and GS nerves respectively instead of PDP. Body temperature 32° C. Same time scale for all records.

In the cool cord Brooks & Koizumi (1956) found that cutaneous afferent volleys caused the discharge of DRRs into muscle afferent fibres and reciprocally that muscle afferent volleys produced DRRs in cutaneous nerves (cf. Eccles & Krnjević, 1959*b*). The present investigation has confirmed these earlier observations. For example, in Fig. 9A, B volleys in the cutaneous afferents, superficial peroneal (SP) and sural (S) evoked

DRRs into the FDHL nerve, and, in Fig. 9C, SP + S evoked a large DRR, though not so large as that produced by the most effective combination of muscle afferents in Fig. 9D. Muscle afferent volleys were less effective in evoking DRRs into cutaneous afferent nerves, but in Fig. 9E, F there were small DRRs both from VC + FDHL + PL + GS, and from PBST + PDP, into sural nerve. In contrast, SP produced a very large DRR (G), which was not appreciably increased by summation with a FDHL + PL + GS afferent volley (H). Possibly the DRRs of E and F were due to Group II

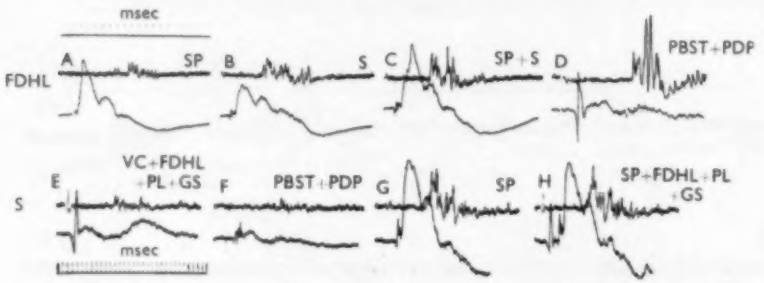

Fig. 9. Interaction between cutaneous and muscle afferent pathways in production of DRRs. A–D, recording of DRRs from FDHL nerve and stimulation of SP, S, SP + S and PBST + PDP as indicated. Time scale above A. E–H same preparations as A–D, but recording from sural nerve (S) and generating single volleys in the various cutaneous and muscle nerves as indicated: time scale below E. Temperature of body, 34·5° C.

afferent impulses from the muscles. Certainly, with DRRs into cutaneous nerves, PBST + PDP do not exhibit the much greater potency that obtains when their Group I afferent volleys produce DRRs in muscle nerves (cf. Figs. 6, 7, and 8). It is possible that it is the Group II components of the muscle nerves that evoke the small DRRs into cutaneous nerves (Fig. 9E, F); further investigation is required.

Type of muscle afferent fibre responding in the DRR

Toennies (1938) showed that DRRs occur in myelinated cutaneous afferent fibres ranging from the largest down to the delta fibres. Brooks & Koizumi (1956) also found that relatively small medullated fibres were concerned both in the inward and outward paths of the DRR. Fibres with conduction velocities below 30 m/sec were apparently not involved in DRRs into mixed cutaneous and muscle nerves such as peroneal or posterior tibial. Three different methods have been employed in attempting to identify the muscle afferent fibres conveying the impulses of the DRR.

(1) The outgoing impulses of the DRR will block afferent impulses travelling in the opposite direction in the same fibres. If an afferent volley

is set up peripherally in the muscle nerve at an appropriate time relative to the discharge of the DRR, there will be a deficit in its spike as recorded by an electrode in contact with the dorsal root. For example, in Fig. 10A the spike produced by a Group I afferent volley from GS nerve was only about half the control size when it was evoked by a stimulus applied 11·7–15·5 msec after a PBST afferent volley; hence the DRR must have discharged along about half of the Group I afferent fibres. When the stimulus to the GS nerve was weakened so that it would be exciting mainly Group Ia fibres (Eccles *et al.* 1957*a*), the spike in the dorsal root was almost completely abolished over the same range of intervals (Fig. 10B).

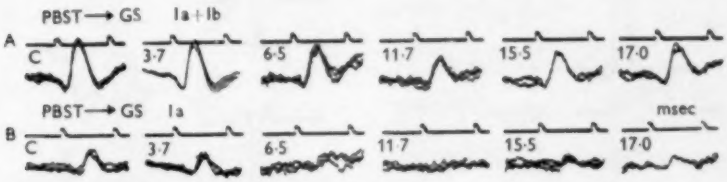

Fig. 10. Blockage of afferent volleys by DRR. In A a stimulus maximal for Group I was applied to the gastrocnemius nerve giving the control spike C in the L7 dorsal root as it enters the cord. In the other records it was preceded by a maximum PBST volley at the intervals indicated in msec. Note the depression of the GS spike at all testing intervals from 6·5 to 17·0 msec, with a maximum depression at 11·7 msec. B is a similar series, but the stimulus to the gastrocnemius nerve was weakened so that it would excite virtually only Group Ia fibres. Body temperature 33·5° C.

Evidently the surviving dorsal root spike in Fig. 10A was in the higher threshold component of Group I, i.e. the DRR was predominantly in the Group Ia afferent fibres of the GS muscle and to a lesser degree in the higher threshold Group Ib fibres. Similar results were invariably obtained in this type of experiment, but it must not, therefore, be concluded that DRRs do not occur in Ib afferent fibres. The threshold discrimination between Ia and Ib afferent fibres is not sufficiently good to justify any such exclusive statement (cf. Eccles *et al.* 1957*a*; Laporte & Bessou, 1957).

(2) An alternative method for identifying the afferent fibres conveying outgoing impulses in the DRR is to record the DRR in them by an intracellular electrode, as has been done previously for cutaneous afferent fibres (cf. Frank & Fuortes, 1955; Koketsu, 1956; Wall, 1959; Eccles & Krnjević, 1959*b*). The origin of the impaled muscle afferent fibre has been determined by stimulating the various muscle nerves and finding the one which directly generated a spike potential. This test invariably gave an unequivocal identification. There was no example of an afferent fibre in the spinal cord that was directly activated from two different muscle

nerves. Eight of the 16 Group Ia fibres sampled by this technique exhibited DRR discharges, as is seen in Fig. 11C–E. Usually there were only one or two discharges, which tended to occur at certain preferred intervals. For example, in Fig. 11E there was a single DRR discharge at the time of the second discharge in C and D. DRRs were also observed in 4 of the 23 muscle afferent fibres that were identified as being in Group Ib both by their high threshold and slightly slower conduction velocity.

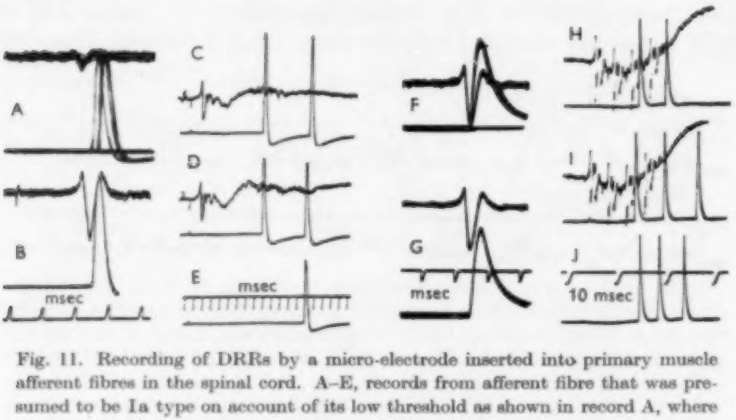

Fig. 11. Recording of DRRs by a micro-electrode inserted into primary muscle afferent fibres in the spinal cord. A–E, records from afferent fibre that was presumed to be Ia type on account of its low threshold as shown in record A, where stimulus just-threshold for the fibre (note failure of spike in many of the superposed tracings) excited also only a small afferent volley, as shown in upper record of A, negativity being recorded downwards. B shows a maximum Group I volley. In C–E are DRRs produced in that fibre by a combined PBST+PDP volley. Dorsal root records are shown, above the intracellular records with their two DRR spikes, in C and D. In E the same volley produced only a single DRR impulse at the time of the second impulses in C and D. Note separate time scales for A, B and for C, D, E. F–J represent a similar series to A–E, but for a presumed Ib afferent fibre from gastrocnemius, because as shown in F its threshold stimulus was almost maximal for Group I, as may be seen by comparing the dorsal root spikes in F and G. In H–J two or three impulses were generated in this fibre by a brief tetanus of 4 PBST volleys at 270/sec. Body temperatures were 31° C for A–E and 36° C for F–J.

For example, in Fig. 11, F and G provide evidence for the identification as a high-threshold gastrocnemius Ib fibre and in H–J repetitive DRRs are seen to be evoked by 4 PBST afferent volleys at 270/sec. In addition DRRs were observed in 5 of the 9 fibres that could have been either Ia or Ib. Three Group II muscle afferent fibres were also impaled and 2 of these showed DRR discharges. It can be concluded that DRRs may occur into any of the large afferent fibres of muscle, though the Group Ia fibres may be predominantly involved.

(3) If the DRR occurs in Group Ia afferent fibres, it would be expected that monosynaptic EPSPs would be produced in the appropriate motoneurones. For example, in Fig. 12B PBST volleys of the strengths and composition indicated in the three tests of Fig. 12A evoked the discharges of DRRs in the medial gastrocnemius nerve. Immediately above these DRRs are intracellular records simultaneously observed from a gastrocnemius motoneurone. It will be seen that motoneuronal depolarization began abruptly less than 1 msec before the onset of the DRR spikes in the peripheral nerve. This is precisely the time relation to be expected for the monosynaptic production of EPSPs by Ia impulses that are generated in the spinal cord close to the presynaptic terminals on the motoneurones.

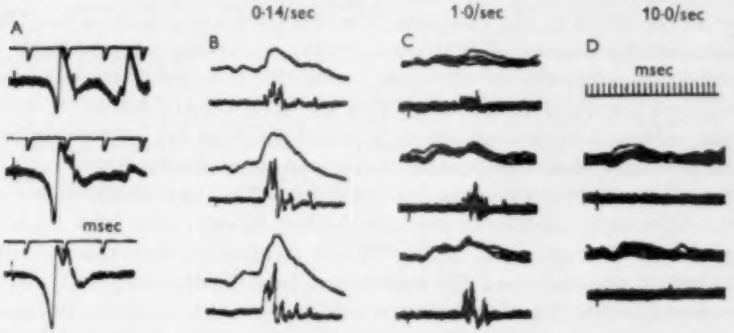

Fig. 12. EPSPs of motoneurones produced by DRRs. In column A are shown tests, as in Fig. 5, for the composition of PBST volleys generated by stimuli of strengths 1·5, 2·1 and 2·8 times threshold respectively. In columns B–D the upper traces are intracellular records from a LG motoneurone, while the lower traces are DRRs recorded from the MG nerve, for these three sizes of afferent volleys (cf. Fig. 5). Note that the principal wave of motoneurone depolarization begins less than 1 msec before the DRR appears in the peripheral nerve (see text). With column B the repetition frequency was 0·14/sec; while with C and D it was 1·0 and 10·0/sec respectively and the records consist of superposed sweeps. Further description in text. Body temperature 34·5° C.

This identification was confirmed by the tests at higher frequency of repetition in Fig. 12C and D. At 1/sec the DRR was much diminished and, correspondingly, so was the motoneuronal depolarization that occurred simultaneously. At 10/sec there was no DRR and the intracellular records show only the small earlier depolarization which also occurred in B and C, and which was probably due to the polysynaptic excitatory action of the PBST afferent volley. This method of testing has certainly confirmed that DRR discharges occur in Group Ia muscle afferents, but so far it has provided no evidence for or against the participation of Group Ib fibres in the DRR.

Recurrent impulse discharges into muscle nerves

When stimulating one muscle nerve and recording from another, it has sometimes been observed that a muscle afferent volley evoked the discharge of an impulse in a muscle afferent fibre by an entirely different mechanism from that producing the DRR. This discrimination was never in doubt, for it was made on the grounds both of the fixed latency of response and of the high frequency at which the discharge followed repetitive afferent volleys, even up to 300/sec or more, whereas the DRR fails even at 10/sec (Fig. 3). A further feature of recurrent discharges is that they often occur reciprocally, i.e. they still appear after reversal of the stimulating and recording nerves. This recurrent discharge may be attributed either to the existence of a branched axon, such as has been postulated by Barron & Matthews (1935) and Habgood (1953), or to ephaptic transmission in the spinal cord. With most of the recurrent discharges the delay in the spinal cord has been about 1·5–2 msec, which is just sufficient for conduction up to and down from the level of the cord section; hence such recurrent discharges are most likely attributable to ephapses made by the cord section (Renshaw & Therman, 1941). However, there have been three examples with central latencies too brief for such a pathway. For example, in Fig. 13 the conduction time was 3·1 msec whether it was measured for conduction from FDHL to PBST (D) or reciprocally (B). The threshold tests A and C show that the recurrent impulse was generated by Ib and Ia afferent impulses in the PBST and FDHL nerves respectively. The shortest conduction times up to the recording electrode on L7 DR at its cord entry were 1·7 msec for the FDHL Group I volley and 1·05 msec for the PBST Ib afferent volley; hence the central delay for the discharge of the recurrent impulse was not more than 0·35 msec. Transmission from one fibre to the other must have occurred close to their entry into the spinal cord, either by some ephaptic contact or by branching. As previously mentioned there was no example of an impaled fibre that could be activated directly from two muscle nerves, but only 51 fibres were impaled, whereas the few recurrent discharges were picked out from observations on many thousands of Group I muscle afferent fibres. The rarity of recurrent discharges with short central latency was also reported by Habgood (1953), there being only two examples in at least 2000 large afferent fibres. Possibly these recurrent discharges occur in collateral branches of primary afferent fibres, as postulated by Barron & Matthews (1935), but the existence of such branches has been doubted on histological grounds (Westbrook & Tower, 1940). A remote possibility that has not been excluded is that these brief-latency recurrent discharges are occurring not in afferent fibres, but in the motor fibres through ephapses that were

made by the ventral root section in the initial preparation (D. R. Curtis, personal communication). It would, however, be surprising if a motor fibre had the very low threshold exhibited in Fig. 13C.

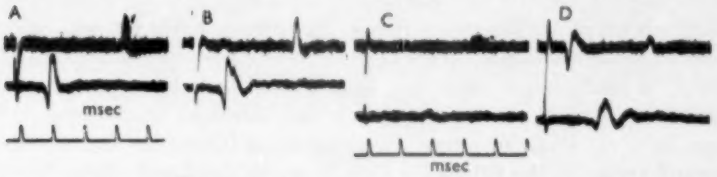

Fig. 13. Recurrent discharges between muscle afferent fibres. In A and B stimuli were applied to PBST nerve and the upper records are from FDHL nerve. With a maximal Group I volley there is a spike in FDHL nerve with a latency of 3·1 msec. As shown in the dorsal root record of A, the threshold stimulus for producing this spike was in the Ib threshold range. Immediately afterwards the stimulating and recording electrodes were reversed and reciprocal conduction with exactly the same latency is seen in D, while in C the threshold for the fibre in the FDHL nerve is seen to be in the low-threshold Ia range. Note that all records are formed by the superposition of many faint traces and there is a small latency range with the just-threshold stimulation in A and C, which is attributable presumably to variations in latency at the site of stimulation. Body temperature 38° C.

DISCUSSION

The demonstration of the widespread incidence of dorsal root reflexes in muscle afferent fibres reveals a hitherto unsuspected richness of interaction between these fibres in the spinal cord. Admittedly in the present investigation this could be fully demonstrated only in the cooled animal. However, the generation of impulses is a threshold phenomenon; in some manner not yet understood (cf. Brooks *et al.* 1955; Brooks & Koizumi, 1956) cooling lowers the threshold for the reflex discharge of impulses from the spinal cord along both ventral and dorsal roots. Thus the DRRs of the cooled preparation give evidence that at normal body temperature there is a subthreshold depolarization of the primary afferent fibres of muscle. Indeed, even at normal or raised body temperature this depolarization occasionally was adequate to generate the discharge of impulses (Figs. 1A–C, G–I; 2A–D; 3I–L). More direct evidence for this depolarization will be provided in later papers, but the present investigation is of value because it demonstrates the existence of the depolarization in the absence of experimental interference with the spinal cord.

The most interesting feature is the much greater effectiveness of the PBST and PDP volleys in producing DRRs. It is not possible to attribute this to the greater population of Group I fibres in the nerve to these flexor muscles. According to Rexed & Therman (1948), Group I fibres form a larger proportion of the afferent fibres of extensor muscles than of flexor

muscles (cf. Lloyd & Chang, 1948), and this preponderance extends also to the proportion measured relative to the total afferent plus efferent fibre population; hence the potency of Group I flexor afferents in producing DRRs must be due, not to numerical dominance, but to the more effective central action of the individual fibres. For example, the Group I afferent volley from the whole assemblage of post-tibial extensor muscles (GS + PL + FDHL) is much larger than that from PBST, and still larger than that from PDP, yet it was always much less effective in evoking DRRs (Figs. 6, 7, 8). Even at a cord temperature as low as 30° C a maximum Group I volley in the GS + PL + FDLH nerves produced either no DRR or a DRR in very few afferent fibres of any other muscle nerve, whether a flexor or extensor. Group I volleys in the nerves to the hip and knee extensor muscles (SMAB and Q respectively) were also relatively ineffective in generating DRRs (Fig. 8E–L). As an approximate estimate, it may be stated that for Group I volleys of the same size a volley from flexor muscles is more than three times as effective in evoking a DRR as that from extensor muscles. Possibly, this relative potency of Group I volleys from flexors is related complementarily to the relative impotence of flexor Group Ib volleys in acting on motoneurones through polysynaptic pathways (Eccles, Eccles & Lundberg, 1957*b*); their central pathway is channelled more to interneurones concerned in the DRR than to those acting on motoneurones. It should be pointed out that the production of DRRs from hip flexor nerves has not been investigated, though investigations of the potentials which such volleys generate in the cord and in the dorsal roots indicate that they would be less effective in evoking DRRs than the knee or ankle flexors (Eccles, Magni & Willis, unpublished observations).

Discussion of the present experimental results on interaction between afferent volleys in the same or different muscle nerves is best postponed until they can be correlated with experimental observations on depolarizations of the Group I afferent fibres in the spinal cord (Eccles, Magni & Willis, unpublished observations). The abrupt cessation of the DRR soon after the onset of a prolonged repetitive stimulation (Fig. 2J–M) is probably attributable in part at least to accommodation of the primary afferent fibre to a continuous depolarization. The depression of successive responses even at repetition rates as slow as 0·2–0·5/sec (Fig. 3) requires a more complex explanation. An important factor probably is the long time needed for full recovery of propagation through interneuronal pathways. It is expedient to defer further discussion to a later paper dealing with the interneuronal pathways. Discussion of the post-tetanic potentiations of DRRs, as illustrated in Fig. 4, will also be postponed to the later paper dealing with the depolarization of primary afferent fibres.

Finally, it can be stated that there is no evidence that DRRs are produced under normal physiological conditions by the discharges of the annulospiral endings or Golgi tendon organs. Their present significance derives from the evidence they provide for central interaction between muscle afferent fibres. It will be shown in subsequent papers that this central mechanism is of great interest and importance in relation to the control of reflex activity of the spinal cord.

SUMMARY

1. Particularly in the cooled animal (cat) muscle afferent volleys evoke reflex discharges into muscle afferents, which in general resemble the dorsal root reflexes (DRRs) of cutaneous afferents.

2. At brief intervals volleys sum in the production of DRRs, but beyond 10 msec there is a prolonged depression, so that rapid repetitive stimulation evokes only a transient DRR and DRRs are depressed when evoked at frequencies higher than 0·2/sec.

3. After prolonged high-frequency stimulation there is usually post-tetanic potentiation for at least 60 sec. Post-tetanic potentiation is also observed after tetanization of the afferent carrying the DRR.

4. Both Ia and Ib afferent volleys are effective producers of DRRs, Groups II and III having little or no action. Knee and ankle flexor afferents are much more effective than afferents from extensor muscles.

5. The DRRs occur equally well into the large afferent fibres of flexor and extensor muscles, but there is evidence that the Group Ia fibres are more involved than the Ib.

6. Preliminary observations show that in the production of DRRs there is also some reciprocal action between cutaneous and muscle afferent fibres.

7. The DRRs are readily distinguished from the very rare recurrent discharges from one afferent fibre to another. These discharges are due either to an ephapse or to branching.

8. The preponderant action of flexor afferent volleys is attributable not to numerical dominance of fibres, but to the more effective action of the individual flexor afferents. There is a preliminary discussion also of other features of the central interaction between muscle afferent fibres.

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CENTRAL INHIBITORY ACTION ATTRIBUTABLE TO PRESYNAPTIC DEPOLARIZATION PRODUCED BY MUSCLE AFFERENT VOLLEYS

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Frank & Fuortes (1957) and Frank (1959 and personal communication) have shown that muscle afferent volleys produce inhibition by diminishing the size of the monosynaptic excitatory post-synaptic potential (EPSP) of motoneurones without having any other demonstrable action on those motoneurones. Thus there was no change in the ionic permeability of the post-synaptic membrane, such as occurs with other types of inhibition (Coombs, Eccles & Fatt, 1955*a*), for there was no associated membrane potential change either at the normal resting potential, or when the membrane potential was altered by a background depolarizing or hyperpolarizing current. Furthermore, there was no associated change in motoneuronal excitability as tested either by the intracellular application of current pulses or by the response to invasion by an antidromic impulse in the motor axon. Evidently it should be concluded that the diminution of the EPSPs is due to a diminished excitatory action of the Group Ia presynaptic impulses. However, Frank (1959) in addition proposed an alternative explanation, which attributed the EPSP depression to an action exerted so far out on the dendrites of the motoneurone that no trace of the inhibitory influence itself could be detected by a micro-electrode in the motoneuronal soma. Since both these alternative explanations would locate inhibitory action at a site remote from the motoneurone soma, Frank (1959) employed the term 'remote inhibition' for the phenomenon. It is proposed here to use the non-committal term 'EPSP depression' instead, until a more appropriate term can be developed later in this paper.

There are many suggestions in the literature that central inhibitory action occurs in the presynaptic pathway, there being block or depression of presynaptic excitatory impulses (Barron & Matthews, 1935, 1938; Renshaw, 1946; Brooks, Eccles & Malcolm, 1948; Howland, Lettvin, McCulloch, Pitts & Wall, 1955). These various suggestions will be examined

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after an account has been given of the present investigation, which confirms and extends the pioneer studies of Frank & Fuortes (1957) and Frank (1959). A preliminary report has already been published (Eccles, Eccles & Magni, 1960*a*), and reference to this work has also been made in lectures (Eccles, 1961*a*, *b*) already published.

METHODS

The experimental procedures conformed to the conventional pattern as reported in previous publications from this laboratory and in the preceding paper (Eccles, Kozak & Magni, 1961), which also may be referred to for the symbols corresponding to the various nerves (see this volume, p. 129). In some experiments the temperature of the animal was kept as low as 34° C, because larger EPSP depressions were observed under these conditions. The temperatures given in this paper were recorded by a thermometer inserted under the scapula and just outside the thoracic cage. Usually the temperature of the oil pool over the cord was 2 or 3° C cooler.

RESULTS

Time course of EPSP depression

The time course of onset of EPSP depression is illustrated in Fig. 1B–D, where a maximum Group I volley in the nerve to posterior biceps and semitendinosus (PBST) preceded at progressively increasing intervals the testing volley in gastrocnemius soleus (GS) nerve that by itself set up the monosynaptic EPSP shown in A. The EPSP depression is seen to increase from a negligible action at a brief interval (2·5 msec in B), to a large effect at 10·2 msec in D. The diminution of the EPSP was not associated with any change in its time course, nor did the conditioning volley produce any appreciable change in the membrane potential of the impaled gastrocnemius motoneurone, as may also be seen in Fig. 2C–E. Measurements from the series partly illustrated in Fig. 1A–D are plotted in E to show all except the final stages of the time course of the EPSP depression.

The depression of the EPSP in Fig. 1 was much larger than that usually observed for a single afferent volley, which often was only 10% or less, as illustrated in Fig. 2A. Much larger depressions are produced by repetitive afferent volleys. For example, 2 volleys at 3·7 msec interval doubled the depression (records of Fig. 2B–E, plotted in F), while with 7 volleys at 270/sec the depression was increased nearly threefold (Fig. 2G), and had a slower time course of decay.

As is shown in Figs. 1E and 2F, G, the onset of the EPSP depression has been gradual, and no precise latency can be given; but usually a significant depression occurred with testing intervals as brief as 5 msec. The maximum depression occurred with test intervals of 15–20 msec. The time course of Fig. 1E would be prolonged on account of the abnormally

low temperature (34·5° C), but even at 37° C (Fig. 2G) the depression persisted beyond 200 msec.

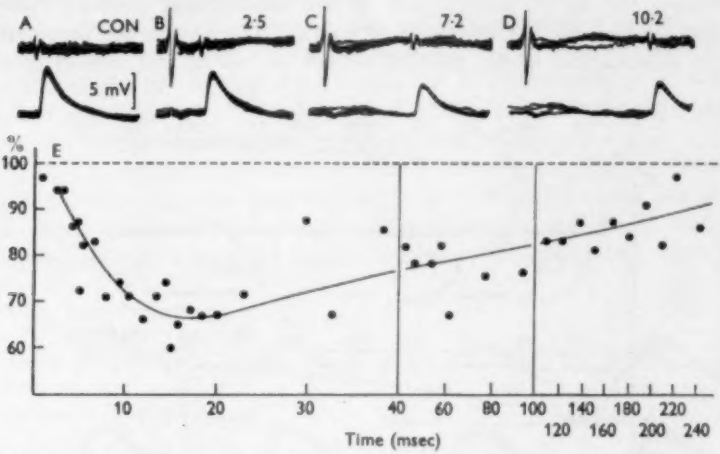

Fig. 1. Time course of EPSP depression. In A–D the lower records give monosynaptic EPSPs intracellularly recorded from a gastrocnemius motoneurone, and evoked by maximal Group Ia volleys in the GS nerve. In the upper records are the potentials recorded from the L 7 DR as it enters the cord, negativity being recorded downwards. All records are formed by superposition of about 5 traces recorded at a frequency of repetition of about 2/sec. A shows the control EPSP, and in B–D the GS volley was preceded by a maximum Group I PBST volley at the intervals marked in msec. The depression of the EPSP was small in B and large at the longer intervals, C, D. The time course of the depression is displayed in E, where the ordinates are the sizes of the EPSPs plotted as percentages of the control EPSPs, and the abscissae the intervals after the conditioning PBST volley. The perpendicular lines denote changes in the abscissal scaling. Body temperature 34·5° C.

Types of afferent impulses giving EPSP depression

In agreement with Frank & Fuortes (personal communication), such investigations as those of Figs. 1 and 2 establish that EPSP depression is due to Group I volleys. It is therefore of particular interest to discover if EPSP depression is due to a specific property of the Ia or of the Ib impulses. For this purpose the PBST nerve was especially suitable, because often an almost complete threshold separation is possible (Bradley & Eccles, 1953; Eccles, Eccles & Lundberg, 1957*a*; Laporte & Bessou, 1957). The experimental investigation is illustrated in Fig. 3, where conditioning volleys in the PBST nerve were evoked by a wide range of stimulus strengths as illustrated by A–D, E–H, I–L and M–O, and their actions tested against the monosynaptic EPSPs produced in a gastrocnemius motoneurone by a maximum Ia GS volley 20·5 msec later (Fig. 3P),

the control EPSPs being shown in B, F, J and N. Immediately after this series the conventional tests were applied (cf. Fig. 3D, H, L) in order to determine the Ia–Ib composition of the conditioning afferent volleys evoked by the various strengths of stimuli. The curves for percentage Ia–Ib composition of the afferent volleys set up by the stimuli of the

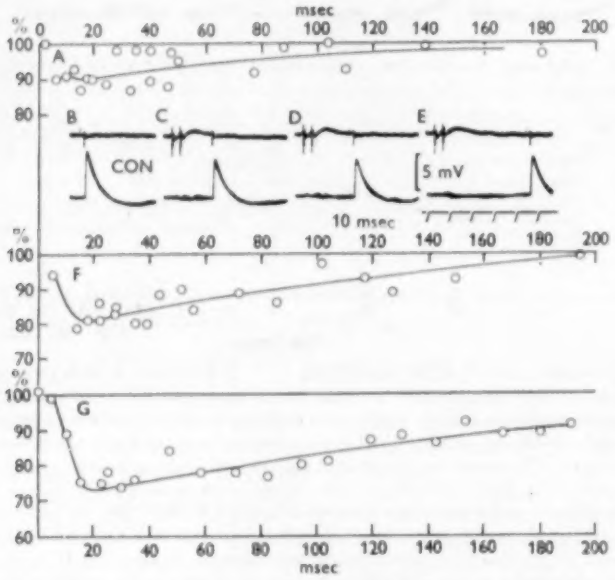

Fig. 2. Time courses of EPSP depression produced by one or more conditioning volleys. Monosynaptic EPSPs produced in a plantaris motoneurone, membrane potential −75 mV, by a maximum Ia plantaris volley and depressed by 1, 2, or 7 maximum Group I PBST volleys. A, plotting of depression by a single PBST volley as in Fig. 1E, but illustrating the much smaller depression usually observed. B–E show records as in Fig. 1A–D, but for depression by two PBST volleys 3·7 msec apart, F showing the plotted points for the whole series. With G there is a similar plotting after conditioning by 7 PBST volleys at a frequency of 270/sec. With all records the frequency of repetition was 0·5/sec. Body temperature was 37° C. With F and G zero time corresponds to first PBST volley.

various strengths are plotted in Fig. 4B, as has been done previously (Eccles *et al.* 1957*a*). When the EPSP depression is plotted on the same abscissal scaling (Fig. 4A), it is seen that almost all of this depression came in at a range of stimulus strengths which corresponded closely to the Ib range, there being only a negligible effect over the Ia range.

However, this almost exclusive relationship of Ib to EPSP depression was unusual, and Fig. 5E shows clearly two steps in the size of the

depression as the conditioning stimulus was increased. The component attributable to the Ib volley was only a little larger than that due to the Ia volley (Fig. 5E). Most of our series have conformed to Fig. 5 in showing that a substantial component of the EPSP depression was due to Ia impulses, and with some the Ia component was even a little larger than the Ib. So far as has been determined, the EPSP depressions produced by Ia volleys (cf. Fig. 5C) were similar in every respect to the depressions produced by the combined Ia–Ib volleys (Fig. 3E–H).

In Figs. 3 and 4A there was no further increase in the EPSP depression when the stimulus was increased to 4 or 8 times threshold, so as to add Group II and III impulses to the conditioning volley. In Fig. 5E it appeared that somewhat larger depression was produced when the

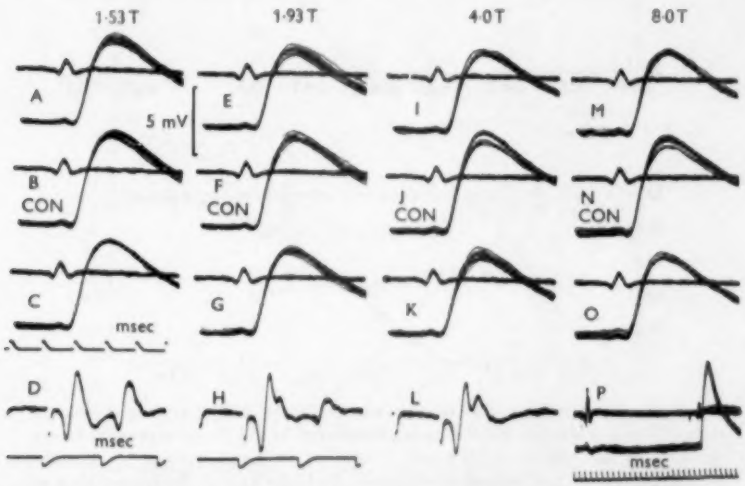

Fig. 3. Type of muscle afferent fibre responsible for EPSP depression. As is shown in P, the conditioning PBST volley preceded the monosynaptic EPSP of the gastrocnemius motoneurone (membrane potential, −80 mV) at a fixed interval of 20·5 msec. The conditioning volley was set up by stimuli of varying strengths up to 8 times threshold (8 T) and specimen records of the conditioned EPSPs (set up by maximum Ia GS volleys) are shown in 4 columns at the indicated strengths: A–C being for 1·53T, E–G for 1·93T, etc. In each series a control EPSP (CON) is shown between two conditioned EPSPs. Note the expanded time scale of these EPSPs relative to that of P. Each record is formed by the superposition of about 10 faint traces at 2/sec. The Ia composition of the PBST conditioning volley used in each vertical column was tested by the double-volley technique (see Eccles *et al.* 1957*a*, and preceding paper, Eccles *et al.* 1961) and the result illustrated in D, H and L. Thus a testing stimulus 0·75 msec after the conditioning found that virtually all Ia and no Ib fibres were refractory in D, while in H about half Ib fibres were refractory and in L all Ia and Ib were refractory.

conditioning stimulus was increased from maximal for Group I (2·2T) to 3·2 and 4·0T, but it may be doubted if this effect is significant. So far there has been no convincing demonstration that Group II and III afferent impulses are effective in producing EPSP depression.

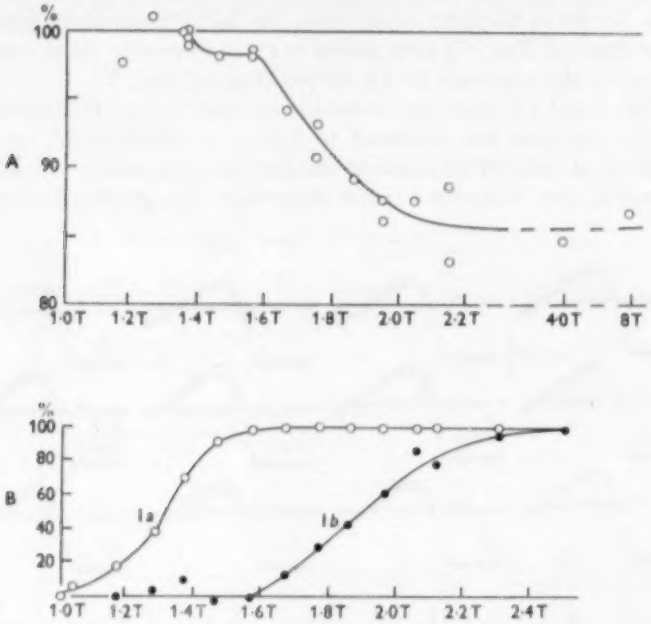

Fig. 4. Types of muscle afferent fibre responsible for EPSP depression. A. The sizes of the conditioned EPSPs partly illustrated in Fig. 3 are expressed as percentages of the control EPSPs and plotted as ordinates against the stimulus strengths relative to threshold as abscissae. In B the sizes of the Ia and Ib components of the conditioning afferent volleys (PBST) were measured from records such as Fig. 3D, H, L, and expressed as percentages of the maximal size, exactly as was done by Eccles *et al.* (1957*a*). Note that the abscissal scalings of A and B are identical.

EPSP depression by repetitive nerve volleys

When the EPSP depression was tested during repetitive stimulation of the conditioning afferent nerve, there was a rapid increase in the depression, as indicated in Fig. 6A, a maximum value being reached with a very few impulses. After cessation of the train of conditioning volleys there was a fairly rapid decline of the EPSP depression, as indicated in the records of Fig. 6B–E, but full recovery could take more than 1 sec (Fig. 6F).

When repetitive stimulation was continued after the maximum EPSP depression had been attained, there was usually a slow decline over several seconds, but eventually the depression tended to stabilize at some intermediate level. For example, Fig. 7 B, C, D illustrates the EPSP depression

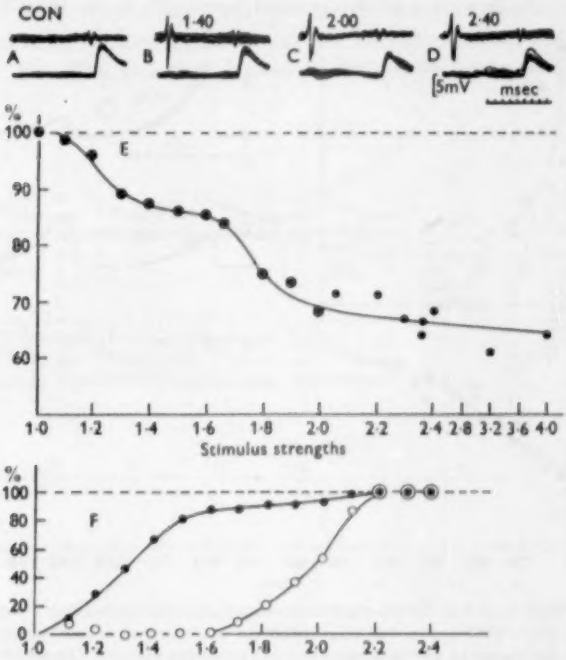

Fig. 5. Types of muscle afferent fibre responsible for EPSP depression. In B–D a conditioning PBST volley was evoked by stimuli of the indicated strengths relative to threshold and followed at a fixed interval (9·0 msec) by a monosynaptic EPSP produced by a maximum Group I a gastrocnemius volley in the same gastrocnemius motoneurone as in Fig. 1. The points in E and F are plotted as in Fig. 4 A, B. Note that in contrast to Fig. 4, a considerable EPSP depression was produced by a Group I a afferent volley, i.e. with stimulus strengths up to 1·6 T. Body temperature 34·5° C. In E the larger plotted points up to 2·0 T are means of two or three records.

at 5, 10 and 20 sec after the onset of repetitive stimulation of the PBST nerve at 300/sec, while Fig. 7 G, H, I gives a similar series for repetitive PDP stimulation. Measurements from these and other similar tests on this same EPSP are plotted in Fig. 7 E and J, and show that the weaker depression produced by the PDP volleys is less well maintained than the

PBST depression. The very large depression in another experiment (Fig. 7K) was well maintained even during 60 sec tetanization.

Summation of the depressant actions of repetitive volleys has been illustrated in Figs. 2 and 6 for high-frequency stimulation at 270 and 210/sec respectively. In Fig. 8A–E the frequency of the conditioning tetanus was varied. As is shown in the plotted curve (F), more than half of the

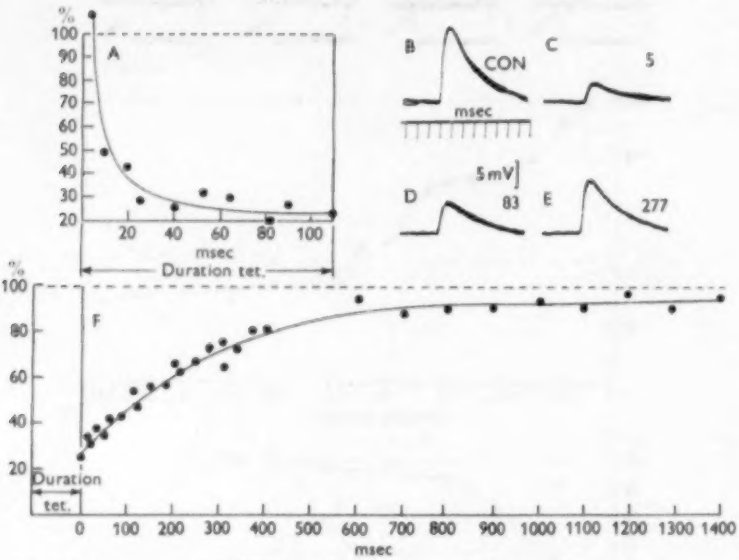

Fig. 6. Time course of EPSP depression during and after repetitive conditioning volleys. The PBST nerve was stimulated for 109 msec at a just-supramaximal strength for Group Ia and at a frequency of 210/sec (22 volleys). The EPSP depression was measured by the size of the maximum monosynaptic EPSP set up by a gastrocnemius volley in a gastrocnemius motoneurone. A shows the rapid increase of the depression during the stimulation. B–E show specimen records (two superimposed traces) of the control EPSP (B) and at the intervals (indicated in msec) after the end of the conditioning tetanus. The time course of recovery from the depression is shown by the plotted points of F for the whole series that was partly illustrated in B–E. In order to allow time for full recovery there was an interval of 2 sec between each conditioning tetanus, and there was, of course, only one testing EPSP after each tetanus. Body temperature 34·5° C.

maximum depression was produced by a frequency of 65/sec, and there was little further increase above 200/sec. Figure 8G–K shows much the same frequency dependence for the very large depression illustrated in Fig. 6.

Besides thus producing a deep and long-lasting EPSP depression, a prolonged tetanization of the conditioning nerve also resulted in a typical

post-tetanic potentiation of the EPSP depression produced by a single conditioning volley. For example, in Fig. 9, following the control records of the depressed EPSP (A) and of the EPSP alone (B), there was a long tetanus of the conditioning nerve. Two seconds later there was still a small residual depression of the EPSP alone in one control response of D (cf. Fig. 6F). The conditioning volley exerted a greatly increased depression (C), as may also be seen at 12 sec (E, F), and even to a small extent at 90 sec (G, H). The full time course of the post-tetanic potentiation of

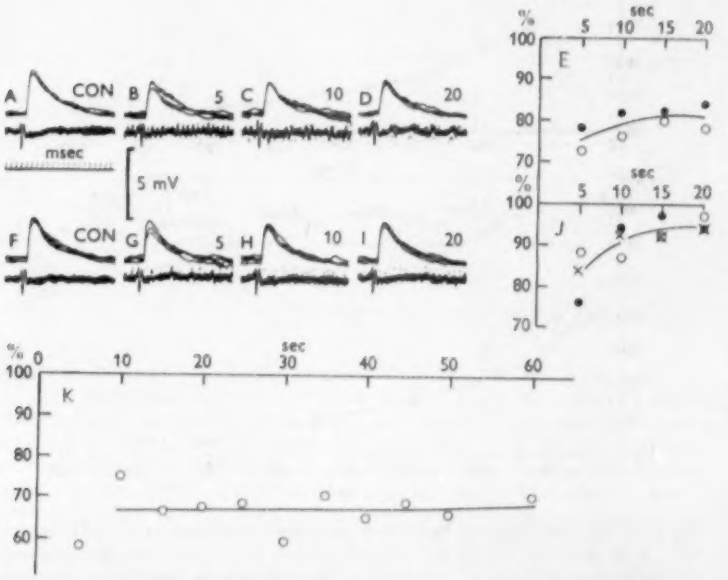

Fig. 7. EPSP depression during prolonged tetanization of the conditioning afferent nerve. The GS nerve was stimulated continuously at 5/sec at a strength supramaximal for Group Ia and the EPSPs were recorded intracellularly from a gastrocnemius motoneurone (membrane potential, −70 mV), there being four superimposed traces in each record of A–D and F–I, A and F being the control EPSPs. After record A the PBST nerve was stimulated at 300/sec at a strength sufficient to maintain it supramaximal for Group I during a prolonged tetanus. After 5 sec conditioning tetanus the EPSPs were still depressed (B), as also at 10 and 20 sec in C and D during this same conditioning tetanus. E gives the plotted points for these records and for one other series taken just afterwards. A similar series of traces for depression of the same EPSP during a PDP tetanus at 300/sec is shown in G–I and plotted in J along with two other series on the same EPSP. It is seen that the depression is less well maintained than during the PBST tetanus. Body temperature 37° C. In K the EPSP depression is seen to be even better maintained than in E. This is also for a PBST tetanus at 220/sec on to an EPSP of a gastrocnemius motoneurone.

the EPSP depressant action (Fig. 9I) exhibited a relative potentiation and a duration comparable with that observed for monosynaptic actions after a similar conditioning tetanus (Eccles, Krnjević & Miledi, 1959; Curtis & Eccles, 1960).

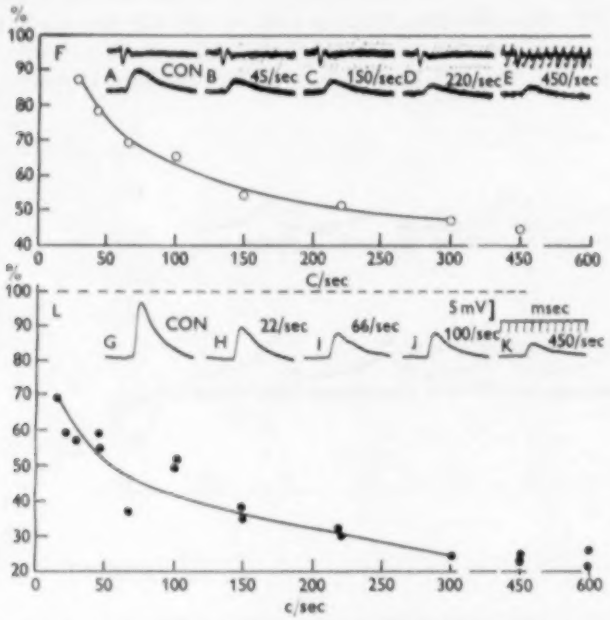

Fig. 8. Effect of frequency of conditioning stimulation on intensity of EPSP depression. A–F, specimen records and plotted curve of EPSP depression produced on the monosynaptic EPSP of a gastrocnemius motoneurone (membrane potential, −35 mV) by maximum Group I PBST volleys at various frequencies. A gives control EPSP and in B–E the EPSP was photographed after a brief duration (about 1 sec) of the conditioning tetanus at the indicated frequencies. Thus the depression was recorded before there had been the decline shown in Fig. 7. The EPSP were recorded at a repetition frequency of about 20/sec, and each record of A–E was formed by the superposition of about 10 faint traces. The traces above the EPSPs show that there was no appreciable change in the sizes of the gastrocnemius afferent volleys as recorded from the L7 dorsal root at cord entry. The plotted points of F are for the series partly illustrated in A–E. Body temperature 37° C. G–K give specimen records of control EPSP and depressed EPSPs at the stated frequencies of conditioning tetani for the same motoneurone as Fig. 6, while L gives the curve for EPSP depression–frequency relationship. Body temperature 34·5° C. Same time scale for A–E and G–K.

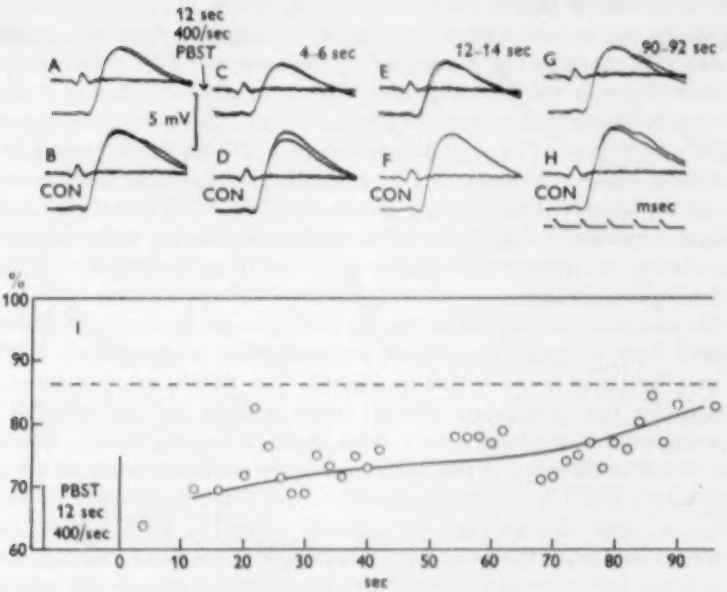

Fig. 9. Post-tetanic potentiation of EPSP depression. A and B illustrate the control level of EPSP depression (A/B) produced by a maximum Group I PBST volley on the monosynaptic EPSP of a gastrocnemius motoneurone (membrane potential, −75 mV). After a conditioning PBST tetanus (of 400/sec for 12 sec) a much larger depression, C/D, was produced by the PBST volley, as also at 12 sec post-tetanically, E/F; but at 90 sec there was recovery (G/H) almost to the pre-tetanic ratio. Testing EPSPs were evoked every 2 sec, there being two superimposed in each trace, and the control EPSPs (D, F and H) were usually taken in alternating sequence. These controls were remarkably steady except for the depression of the first one after the conditioning tetanus (one record of D). The series partly illustrated in A–H is fully plotted in I to show the time course of the post-tetanic potentiation of the EPSP depression.

EPSP depression in relation to the various species of muscle

Hitherto EPSP depression has been illustrated and discussed in relation to the actions of PBST volleys on monosynaptic EPSPs of gastrocnemius and plantaris (Fig. 2) motoneurones. With this arrangement there is the advantage of having large depressions, and in addition the tests are generally free from complications by the various actions of Group I afferent volleys that occur by the conventional synaptic pathways. PBST Group Ia volleys usually exert no excitatory or inhibitory action on GS and PL motoneurones (Eccles, Eccles & Magni, 1960*b*), and in only about one quarter of gastrocnemius motoneurones have Group Ib PBST volleys

any excitatory or inhibitory action (Eccles, Eccles & Lundberg, 1957*b*). By taking advantage of these favourable conditions, it has been possible to establish many of the distinguishing features of EPSP depression; as a consequence it can be recognized even when the conditioning volleys exert post-synaptic inhibitory or excitatory actions on the motoneurone under observation. The very long duration of the EPSP depression is of particular value in this respect. It is justifiable to assume that no other Group I actions continue beyond 25 msec in the isolated spinal cord under sodium pentobarbital anaesthesia. Furthermore, during rapid repetitive stimulation the polysynaptic actions of Group Ib volleys rapidly diminish and even disappear.

The simplest method of surveying the relative potencies of afferent volleys from different muscles in depressing the monosynaptic EPSPs of motoneurones is illustrated in Fig. 10. The EPSPs are generated by stimulating the appropriate afferent nerve at 5/sec and are recorded by superimposition of about 3 or 4 faint traces. The conditioning nerve is stimulated at 300/sec and the EPSPs are again recorded for about 1·9 sec, beginning a fraction of a second after the onset of the conditioning tetanus, which is itself discontinued immediately after the EPSPs have been photographed. In this way full advantage is taken of the optimum conditions for detecting any EPSP depression that may be present. There is of course a risk that post-synaptic inhibitory actions might depress the EPSP, as described by Coombs, Eccles & Fatt (1955*b*) and by Curtis & Eccles (1959), but this effect rapidly declines during repetitive stimulation at 300/sec, though some Group Ia inhibition may not be thus eliminated.

Table 1 summarizes the effects thus observed on monosynaptic EPSPs in seven motoneurones in one experiment. Invariably the PBST volley was the most effective, but the P and DP volleys were also surprisingly potent. When their relatively small sizes are taken into account, they must be at least as effective as the PBST volleys. With all the other nerve volleys the EPSP depression was either absent or so small as to be negligible. In other experiments GS and Q (quadriceps) volleys have produced significant depressions. The most significant features of Table 1 are the EPSP depressions produced by PBST, P and DP volleys in all motoneurones. This has been the general rule; but much more systematic examination is needed before the full pattern of action is disclosed.

Correlation of EPSP depression with dorsal root reflexes

It will be sufficiently obvious that there is a very close parallelism between the investigations on the dorsal root reflexes (DRR) in muscle afferent fibres as reported in the preceding paper (Eccles *et al.* 1961) and

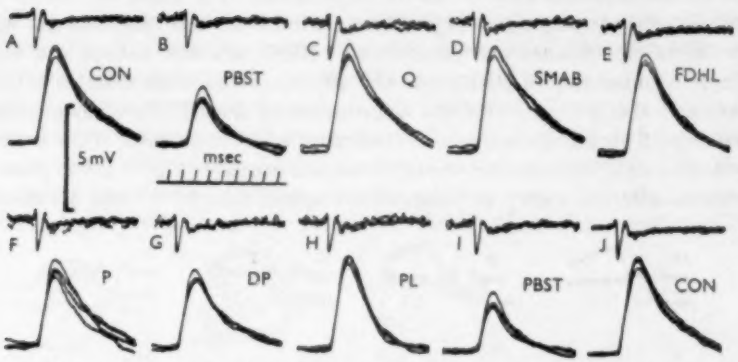

Fig. 10. Monosynaptic EPSPs during conditioning by repetitive stimulation of many different muscle afferents. The EPSPs were generated in a gastrocnemius motoneurone (membrane potential, −66 mV) by maximum Group Ia GS volleys at a repetition rate of 5/sec and each record is formed by the superposition of 3 or 4 traces. This repetitive GS stimulation was continued throughout the whole series. In B–I various muscle nerves, as indicated by the symbols, were stimulated at 300/sec and just-supramaximally for Group I. About 1 sec after the onset of this tetanization several EPSPs were recorded superimposed, the conditioning tetanus being then changed to some other nerve and the EPSPs again recorded. Thus B–I show the sequence of observations with the various conditioning tetani and A and J the control EPSPs before and after the series. Note that, as is shown by the records above the EPSPs, there was no sign of depression of the GS afferent volley as recorded from L7 dorsal root at its cord entry, negativity being downwards. Body temperature 37° C.

TABLE I

	RP (mV)	Conditioning nerves: PBST	P	DP	SMAB	PL	FDHL	GS	Q
GS	−58	72	85	91	100	—	—	—	100
	−66	60	79	79	—	104	96	—	102
	−30	50	66	82	100	100	—	—	—
	−35	48	66	80	98	91	—	—	—
PL	−75	68	86	84	99	—	100	99	98
P	−59	42	—	68	96	100	—	96	—
PBST	−77	—	83	84	98	101	—	98	—
Means		57	78	81	98·5	99	98	98	100

The numbers give the sizes of the monosynaptic EPSPs of the various motoneurones listed to the left when subjected to brief conditioning tetanization at 300/sec of the nerves shown above each column. The sizes are measured relative to the mean values of the control EPSPs recorded before and after each series of conditioning tetanizations. Many of the percentages are means of several determinations. Specimen records are shown in Fig. 10 for the EPSPs of the second GS motoneurone. Column labelled RP shows membrane potential.

the results reported here for EPSP depressions. For example, Group Ia and Ib afferent impulses conjointly contribute to both phenomena, and the fields of influence are parallel, e.g. PBST afferent volleys are very effective generators of DRRs into the afferent fibres of all muscles of that limb and also are very effective depressants of the EPSPs of their motoneurones. This parallelism is well illustrated when two effects of the testing gastrocnemius stimulus are recorded simultaneously (Fig. 11): the gastrocnemius afferent volley as it enters the spinal cord in S1 and L7 dorsal

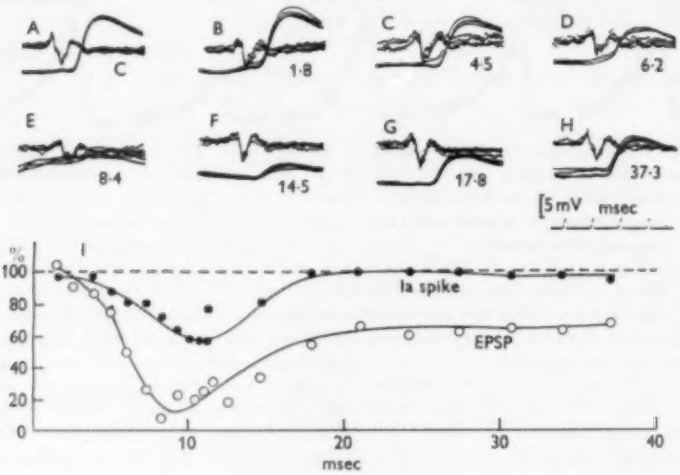

Fig. 11. Relation of EPSP depression to depression of testing afferent volley. In A–H monosynaptic EPSPs are recorded intracellularly in a gastrocnemius motoneurone and the gastrocnemius afferent volley is also recorded from L7 dorsal root at cord entry, negativity being recorded downwards. A is the control EPSP and in B–H it is preceded by a maximum Group I PBST volley at the intervals indicated in msec. Each record is formed by superposition of 3 or 4 faint traces at a repetitive frequency of 0·5/sec. The sizes of the EPSPs as percentages of the control are plotted as open circles in I. The sizes of the spike potentials produced by the gastrocnemius afferent volleys are measured between the initial positive and negative spike summits, are calculated as a percentage of the control and are plotted as filled circles in I. Body temperature 34·5° C.

roots; and the monosynaptic EPSP which it produces in a gastrocnemius motoneurone. Both were diminished by the conditioning PBST volley in Fig. 11 D, E, but, as is shown in the plotted points of Fig. 11 I, the afferent spike potential had fully recovered at 18 msec test interval, whereas the EPSP was depressed almost to 60% at the longest test interval. Possibly the very large EPSP depression in Fig. 1 E at testing intervals up to 20 msec is in part due to diminution of the testing pre-

synaptic volley by the DRR, just as in Fig. 11I. When the stimulus to the GS nerve was weakened so that it virtually excited only Group Ia afferent fibres (Fig. 12A–E), the proportional depression of the gastrocnemius afferent volley was greater, as reported in the previous paper (Eccles *et al.* 1961), but it was still less than the EPSP depression. Evidently in Fig. 11I two factors were concerned in the depression of the EPSP at intervals up to 18 msec: diminution of the Ia afferent volley on account of block of incoming impulses by collision (or later refractoriness) with impulses discharged out along the Ia fibres, i.e. by the DDR; and depression of the

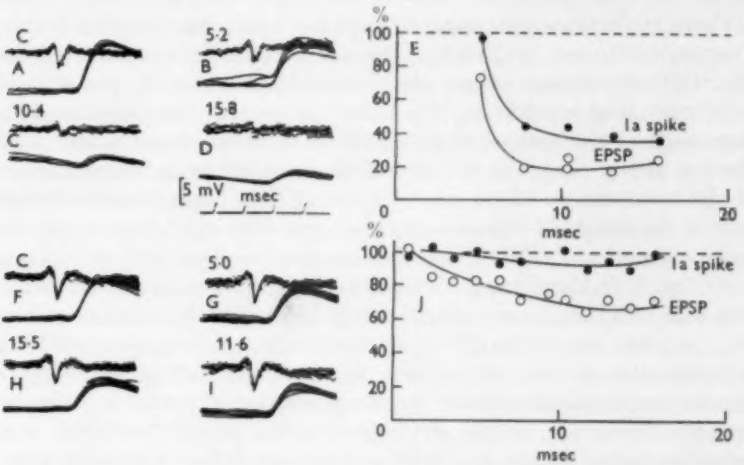

Fig. 12. Relation of EPSP depression to depression of the afferent volley. Continuation of the series of Fig. 11. The stimulus to the gastrocnemius nerve was diminished until it was submaximal for Group Ia, A being the control as in Fig. 11A, while B–D resemble Fig. 11B–H, being recorded at the indicated intervals after the same conditioning PBST volley, at a repetition rate of 0·5/sec. Measurements from the series partly illustrated in A–D are plotted in E with the same symbols as in Fig. 11I. F–I resemble Fig. 11A–H, F being the control and G–I at the test intervals indicated in msec, except that the frequency of repetition was raised to 5/sec, so as virtually to eliminate complications due to dorsal root reflexes (Eccles *et al.* 1961). Measurements from the series partly illustrated in F–I are plotted in J with the same conventions as in E. Further explanation in text.

EPSP produced by the remaining fraction of the Ia volley. This inference is supported by observing the effect of a tenfold increase in repetition rate, from 0·5/sec in Fig. 12A–E to 5/sec in Fig. 12F–J. At such frequencies the DRRs in muscle afferent fibres are virtually eliminated (Eccles *et al.* 1961), and correspondingly there was no more than a 5% depression of the Ia spike potential of the testing afferent volley (Fig. 12G), yet the EPSP

was still depressed to below 70%. The large EPSP depressions produced by prolonged high-frequency (200/sec or more) stimulation of the conditioning muscle afferent nerve (Figs. 6, 7, 8) certainly establish that the EPSP depression occurs independently of a peripheral block of the incoming testing afferent volley. Under such conditions there would be no DRRs after the initial discharge of about 20 msec duration (Eccles *el al.* 1961), so there would be no diminution of the testing afferent volley by collision with DRR discharges; yet the largest EPSP depressions occurred under such conditions.

DISCUSSION

There has been no systematic attempt to confirm the report by Frank & Fuortes (1957) and by Frank (1959 and personal communication) that the EPSP depression is not associated with any change in the post-synaptic membrane that is exhibiting the EPSP. However, it can be stated that depression of the monosynaptic EPSP of a motoneurone occurs in the absence of any change in the membrane potential, or in the excitability, of the motoneurone which has been tested both by antidromic invasion and by the threshold depolarization for generation of an impulse; furthermore, even when the EPSP was depressed to less than 25% of its control size (Figs. 6, 8), there was no detectable change in its time course, and this also indicates the absence of any change in the post-synaptic membrane. On the other hand the EPSP depression is precisely correlated with depolarization of the presynaptic fibres. When sufficiently large this depolarization manifests itself by the generation of impulses in the presynaptic fibres, i.e. by the dorsal root reflex (DRR) described in the preceding paper (Eccles *et al.* 1961). However, it has been more directly displayed by intracellular recording from the primary afferent fibres and by their enhanced excitability as tested by brief current pulses (Eccles, Magni & Willis, unpublished observations). In every experimental test that has been applied there is excellent correlation between this presynaptic depolarization and the EPSP depression; hence it can be concluded that EPSP depression is a manifestation of a presynaptic mechanism of inhibition. It is appropriate therefore to designate this inhibitory phenomenon as 'presynaptic inhibition', which was the term originally used by Frank & Fuortes (1957).

It is postulated that presynaptic depolarization results in EPSP depression because it depresses the size of the presynaptic impulse and hence decreases the liberation of excitatory transmitter substance. A very steep relationship between size of presynaptic impulse and the EPSP was demonstrated by Hagiwara & Tasaki (1958) with the giant synapse of the squid stellate ganglion, where there was virtually complete suppression of the EPSP when a depolarizing current depressed the presynaptic

impulse to 80% of its normal size, while an increased presynaptic spike gave a great increase in the EPSP. More indirect evidence led Liley (1956) to postulate that at the rat neuromuscular junction the rate of quantal liberation of transmitter was increased tenfold for every 15 mV of presynaptic depolarization. On such a relationship a 5 mV diminution of the presynaptic spike would reduce the liberation of transmitter to 45%, which would account for all but the largest EPSP depressions reported above. Evidently it is of the greatest importance to investigate the effect of presynaptic depolarization on the size of the presynaptic spike and on the output of transmitter by that spike. There is much more evidence for the opposite action of presynaptic hyperpolarization in greatly increasing the output of transmitter: at the giant synapse of the squid stellate ganglion (Hagiwara & Tasaki, 1958); at the frog neuromuscular junction (del Castillo & Katz, 1954); and with monosynaptic EPSPs in the cat cord, where it provides at least part of the explanation of post-tetanic potentiation of monosynaptic reflexes and EPSPs (Lloyd, 1949; Eccles & Rall, 1951; Eccles & Krnjević, 1959).

It may be provisionally concluded that the EPSP depression is fully explained by the presynaptic depolarization together with the blockage that occurs during the outward passage of the impulses of the DRR. Of course such impulses in Group Ia fibres of the testing muscle nerve will themselves produce monosynaptic EPSPs that precede the EPSP produced by the remaining impulses of the testing afferent volley. Such an EPSP is seen in Fig. 11 D–F, where the testing EPSP was superimposed on an earlier EPSP in D–E and occurred during its decline in F, as also may be seen in Fig. 12C, D. Other examples are shown in the preceding paper (Eccles *et al.* 1961, Fig. 12B, C).

A full discussion of the production of presynaptic depolarization cannot be attempted until the more direct investigations have been reported. There have been many previous suggestions that inhibition is brought about by some presynaptic mechanism. In almost all cases it has been attributed to blockage of the presynaptic impulses by the flow of currents that exert either a hyperpolarizing or depolarizing action on the branching presynaptic fibres (Barron & Matthews, 1935, 1938; Renshaw, 1946; Brooks *et al.* 1948; Howland *et al.* 1955). In part this earlier experimental evidence for presynaptic blockage is explained by the block produced by DRRs as illustrated here (Figs. 11, 12; Eccles *et al.* 1961, Fig. 11) and previously suggested (Brooks *et al.* 1948). This latter investigation is of particular interest in relation to the present findings. Focal recording of presynaptic spikes and monosynaptic EPSPs showed that at a sufficiently long interval after the conditioning volley there was no block by DRR discharge, but there was a reduction in size of the presynaptic spike in

the motoneuronal nucleus and a corresponding reduction in the EPSP. This effect was attributed to presynaptic depolarization blocking the presynaptic impulses close to their synaptic terminals. It now seems likely that this was an example of a true presynaptic inhibition and that the presynaptic depolarization was displayed both by the diminished negative phase of the presynaptic spike and by the diminished EPSP. Other observations in that paper may now be interpreted as examples of EPSP depression that were due to presynaptic inhibition. The monosynaptic EPSPs exhibiting this depression were recorded either focally by an extracellular micro-electrode, or after electrotonic spread to the ventral root.

When produced by repetitive volleys presynaptic inhibition causes a remarkable depression in the sizes of testing EPSPs, as is impressively shown in Figs. 6 and 8. It would be expected that under such conditions there would be a deep prolonged inhibition of reflexes, and this has been observed. The long duration of the presynaptic inhibition produced by a single volley gives opportunity for summation of the asynchronous discharges from muscle receptor organs; hence it would be expected that through their presynaptic inhibitory action such discharges would be very effective in depressing reflexes.

SUMMARY

1. There has been detailed investigation of the finding of Frank and Fuortes that a muscle afferent volley depresses the size of the monosynaptic EPSP produced by another muscle afferent volley in a motoneurone that otherwise is unchanged in its responses.

2. EPSP depression occurs with a testing interval as brief as 5 msec; it reaches a maximum in 10–20 msec and slowly decays, having a total duration of 200 msec or more.

3. EPSP depression is produced by both Group Ia and Ib afferent impulses, and little if at all by Group II and III afferent impulses.

4. During prolonged repetitive stimulation of the conditioning nerve, the EPSP depression reaches a maximum after the first few impulses and there is subsequently a partial decline, though it may be well maintained for 60 sec. With increases in frequency up to 100/sec there is a commensurate increase in EPSP depression, but there is little further increase as the frequency rises above 200/sec. Following prolonged tetanization there is a post-tetanic increase in the EPSP depression produced by a test volley; it persists for as long as 60 sec.

5. Afferent volleys from knee and ankle flexors give potent depression of the monosynaptic EPSPs of all motoneurones, whereas volleys in extensor afferents are always much less effective.

6. There is good correlation between the conditions producing dorsal root reflexes (DRRs) in muscle afferent fibres and those giving the EPSP depression, but the latter is not due to blockage of the testing volley by centrifugal impulses of the former.

7. It is concluded that both the DRR and the EPSP depression occur on account of depolarization of presynaptic fibres in the spinal cord; hence EPSP depression and the resultant depression of reflexes is designated 'presynaptic inhibition'.

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IDENTIFICATION OF ACETYLCHOLINE, 5-HYDROXY-TRYPTAMINE, HISTAMINE, AND A NEW KININ IN HORNET VENOM (*V. CRABRO*)

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The venom of the common wasp, *Vespa vulgaris*, contains 5-hydroxytryptamine (5-HT), histamine and a kinin (Jaques & Schachter, 1954; Holdstock, Mathias & Schachter, 1957). The present experiments demonstrate that, like that of the wasp, the venom of the European hornet, *V. crabro*, contains 5-HT and histamine. It differs from that of the wasp, however, in that it contains high concentrations of ACh and also a kinin which is not identical with the one in wasp venom.

In recent years evidence has accumulated of the existence in nature of pharmacologically active choline esters other than ACh, such as propionylcholine (PrCh) in ox spleen (Banister, Whittaker & Wijesundera, 1953), urocanylcholine (Murexine) and β-β-dimethylacrylylcholine (DMAC) in the hypobranchial glands of certain molluscs (Erspamer & Benati, 1953; Keyl, Michaelson & Whittaker, 1957). An active ester of choline, closely resembling or identical with DMAC, has also been found in the cervical (defensive) gland of the Garden Tiger moth, *Arctia caja* (Bisset, Frazer, Rothschild & Schachter, 1960). The choline ester in hornet venom was therefore carefully examined by chemical and pharmacological tests and was identified as ACh.

The kinin in hornet venom is classified as such because it contracts the isolated rat uterus and guinea-pig ileum, relaxes the rat duodenum, and lowers the arterial blood pressure of the rabbit. It has been distinguished from oxytocin, wasp kinin and bradykinin, but closely resembles the latter.

A preliminary report of this work has been communicated to the Physiological Society (Bhoola, Calle & Schachter, 1960).

METHODS

Hornet venom. A nest of live hornets was kindly given to us by Mr R. L. Ford, F.R.E.S. The hornets were frozen at $-10°$ C, then thawed, and the venom apparatus of the females removed by pulling gently on the sting with fine forceps. The venom sacs were dissected free of adjacent tissue, accessory glands and stinging apparatus, and dried *in vacuo* over P_2O_5.

* M.R.C. Scholar.

Extracts. Dried venom sacs (1–5 at a time) were extracted five times with hot ethyl alcohol (95 %). The first extraction was with 1·0, 0·5 or 0·25 ml. (depending on the number of sacs extracted), and the subsequent ones with 0·25 ml. The extract and residue were reduced to dryness *in vacuo*. For assays without preliminary chromatography sacs were extracted with boiling, acidified (HCl) Tyrode solution (pH 3–4) and centrifuged, and the supernatant solution neutralized and tested.

Isolated organ preparations. The isolated guinea-pig ileum, rat duodenum or rat uterus was suspended in a 17 ml. bath in Mg-free Tyrode solution (%: NaCl, 0·8; KCl, 0·02; $CaCl_2$, 0·02; NaH_2PO_4, 0·005; $NaHCO_3$, 0·1; glucose, 0·1) and contractions were recorded with a frontal lever writing on a smoked drum. The bath temperature was adjusted to 35, 30 and 26–28° C for the guinea-pig ileum, rat duodenum and rat uterus respectively. Atropine (10^{-6} g/ml.), mepyramine (10^{-6} g/ml.) and lysergic acid diethylamide (LSD; 10^{-7} g/ml.) were added to the bath when required.

The rectus abdominis muscle of the frog (*Rana temporaria*) was suspended in an 8 ml. bath containing eserinized (5×10^{-6} g/ml.) Ringer's solution at room temperature. Acetone was added to the bath before each test to give a concentration of 1:4000 (v/v); this was found to increase the sensitivity to ACh five- to tenfold. D-Tubocurarine (10^{-6} g/ml.) was added when required.

Blood pressure. Rabbits were anaesthetized with a solution of sodium phenobarbitone (60 mg/kg) and urethane (500 mg/kg) (= 2 ml./kg) injected intravenously, and blood pressure was recorded from the carotid artery with a mercury manometer. Test substances were injected through a cannula in the external jugular vein and washed into the circulation with a constant-volume injection apparatus.

Paper chromatography. Ethyl alcohol (95 %) extracts of 1–6 venom sacs were dried, redissolved in 50 % ethyl alcohol and applied as single spots (equivalent of 0·5–1·5 mg dry venom sac) or as a 5 cm strip (equivalent of 10 mg dry venom sac) on the paper. Markers of acetyl-, butyryl-, and propionylcholine, choline, histamine and 5-HT (25 or 50 μg) were also applied as spots. The ethyl alcohol-insoluble material in the sac was dissolved in a small volume of distilled water, centrifuged, and applied as a 5 cm strip (equivalent of 10 mg dry sac).

Ascending chromatograms were run overnight at room temperature (16–20° C) on Whatman No. 1 paper. The solvents used were: *n*-butanol:ethanol:acetic acid:water (8:2:1:3) (Augustinsson & Grahn, 1953); *n*-butanol:acetic acid:water (4:1:5) (Partridge, 1948); *iso*propanol:ammonia:water (20:1:1) (Smith, 1958); *n*-propanol:formic acid:water (8:1:1), *n*-butanol:water (9:1), and *n*-propanol:water (9:1) (Whittaker & Wijesundera, 1952); *iso*propanol:0·1 N-HCl (7:3) (Lembeck, 1954), and ethanol:0·2 N-HCl (1:1) (Holdstock *et al.* 1957).

Chromatograms were examined under ultra-violet light and fluorescent or quenching areas outlined. Development was by (*a*) Iodine vapour, (*b*) Pauly's reagent: diazotized 1 % (w/v) sulphanilamide in *n*-butanol followed by 50 % saturated sodium carbonate, (*c*) Ehrlich's reagent: 2 % (w/v) *p*-dimethylaminobenzaldehyde in 5 % hydrochloric acid, (*d*) Ninhydrin-acetic acid: 0·25 % (w/v) ninhydrin in acetone (9 parts) in acetic acid (1 part); paper examined under U.V. light (Jepson & Stevens, 1953), (*e*) Ninhydrin-pyridine: 0·25 % (w/v) ninhydrin in acetone plus several drops of pyridine; paper examined under U.V. light, (*f*) Shepherd's reagent: 0·1 % potassium dichromate in 35–40 % formaldehyde, (9/1, v/v); after spraying, heat for 5 min at 110° C and examine under U.V. light (Shepherd & West, 1953), (*g*) Phosphomolybdic acid-stannous chloride: 2 % (w/v) phosphomolybdic acid in ethanol/chloroform (1/1, v/v) followed by 10 % (w/v) stannous chloride in 3 N-hydrochloric acid (cf. Block, Durrum & Zweig, 1958).

For bioassay the paper was cut into horizontal strips beginning 1 cm on either side of the origin, and up to the solvent front as strips 1 or 2 cm wide. Each section was eluted with 1·5–3·0 ml. warm Tyrode solution and 0·05–0·5 ml. eluate was tested on the isolated test preparation.

Ultra-violet absorption spectrum. The area on chromatograms (*n*-propanol:formic acid: water) which corresponded in R_F value and colour reactions to 5-HT was eluted in 3·0 ml. water and its absorption spectrum read in 1·0 cm quartz cells in the Hilger 'Uvispek' against a blank eluted from an adjacent area of the chromatogram exposed only to the solvent.

Drugs and other reagents. Histamine acid phosphate, 5-HT creatinine sulphate, choline, acetyl-, and propionylcholine chloride, butyrylcholine perchlorate, eserine alkaloid, atropine sulphate, mepyramine maleate and D-tubocurarine chloride were the drugs used. The weights of histamine and 5-HT are expressed as base.

Trypsin and chymotrypsin were crystalline preparations (Armour).

Bradykinin was prepared by the action of crystalline trypsin on heated ox serum globulin (Holdstock *et al.* 1957) and contained 140–900 units/mg; wasp kinin was prepared from wasp venom (Holdstock *et al.* 1957).

RESULTS

Initial attempts at bioassay and specific identification of substances in hornet venom sacs indicated that four or more distinct activities were present. Even with specific antagonists of cholinesters, of histamine or of 5-HT, it was not possible to be confident of quantitative assay of more than two substances in the extract. For example, in tests on the isolated guinea-pig ileum it was evident that the major activity could be assayed and antagonized by atropine; similarly, the histamine in the venom could then be assayed on the same preparation in the presence of atropine and its action antagonized by mepyramine. The assay of the remaining activities was difficult, however, because the concentration of ACh-like activity in the extracts was so great, that increased concentrations of atropine were required in attempts to assay the atropine-resistant activity. It was therefore necessary first to separate the active substances by paper chromatography.

After unsuccessful attempts at separation by chromatography in several solvents, we found that a distinct separation of three substances, chromatographically and pharmacologically identical with ACh, 5-HT and histamine respectively, was possible by using *n*-propanol:formic acid: water as a solvent (Fig. 1). However, since the area on this chromatogram corresponding to 5-HT in R_F value and colour reactions was only partially referable to 5-HT in pharmacological tests (approximately one-half of the activity of the eluate was antagonized by LSD), this eluate was re-chromatographed. It was eventually successfully resolved into two components, one identical with 5-HT, the other having the properties of a kinin. The most effective solvent for this purpose was ethanol:0·2 N-HCl (1:1).

Acetylcholine

Activity of extracts on isolated guinea-pig ileum. The ACh-like activity in extracts (0·9 % NaCl + HCl; pH, 3–4) could be assayed on the guinea-pig ileum because the high concentrations present permitted the assay of small amounts of extract, the activity of which was completely antagonized

by atropine in low concentrations (10^{-8} g/ml). The ACh-equivalent concentrations in 4 dry venom sacs tested in this way were 18, 33, 40 and 50 mg/g respectively. One sac extracted with 95 % ethyl alcohol contained 18 mg/g (Table 1).

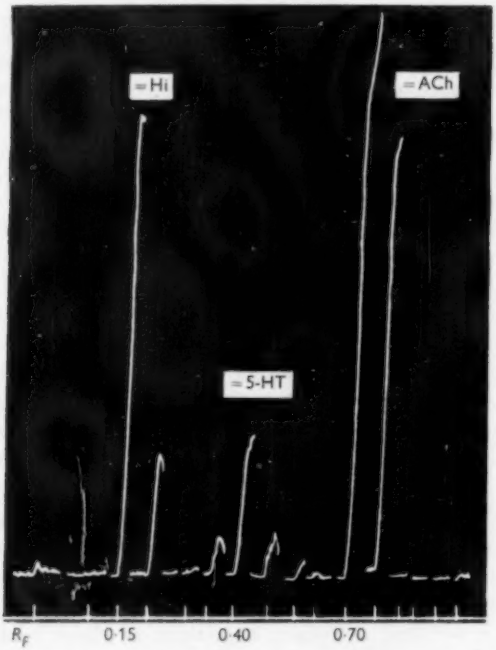

Fig. 1. Contractions of guinea-pig ileum to eluates from a chromatogram (*n*-propanol: formic acid: water) of alcohol extract of hornet venom sac. Three separate activities are eluted which correspond to histamine (Hi), 5-HT and ACh in R_F values, colour reactions and specific antagonism. The eluate corresponding to 5-HT was, however, incompletely antagonized by LSD when tested on the isolated rat uterus, and in fact also contains a kinin which can be separated in another solvent (see Fig. 5).

TABLE 1. Concentrations (mg/g dry weight) of ACh, histamine and 5-HT in hornet venom sacs

Weight of sac (mg)	ACh	Histamine	5-HT	Method
2·8	50	30	—	Acid extract
2·0	33	17	—	
2·2	40	20	—	
3·0	18	14	—	
3·0	22	18	—	Ethanol extract
2·7	10	3	7	Chromatogram eluate
2·8	—	—	15	
8·0 (3 sacs)	—	—	19	

Chromatography, elution and bioassay. As there is now evidence that different, active cholinesters exist in nature (see Discussion) it was necessary to identify the ACh-like substance more specifically. Alcoholic extracts of 1–3 venom sacs were therefore applied as 3–6 spots about 2·5 cm apart on the same paper and chromatographed. After chromatography in *n*-propanol:formic acid:water solvent some of the chromatograms were sprayed with iodine or phosphotungstic acid–stannous chloride reagent, and another similar one was cut into small horizontal strips (see Methods), which were eluted. One portion of each eluate was tested on the isolated guinea-pig ileum and another on the frog rectus abdominis muscle. In every case the eluate which corresponded to ACh in R_F value contracted both the guinea-pig ileum and the frog rectus muscle, the former effect being antagonized by atropine (10^{-8} g/ml.) and the latter by D-tubocurarine (10^{-6} g/ml.). Although the eluates of several distinct areas on the chromatogram contracted the guinea-pig ileum (Fig. 1), the activity of only one was blocked by atropine, and only it contracted the frog rectus muscle (Fig. 2).

To obtain further evidence of the identity of this substance with ACh 10 hornet venom sacs weighing 21·6 mg were extracted with ethyl alcohol and chromatographed as a narrow strip 2·5 cm long in *n*-propanol:formic acid:water solvent, together with separate marker spots of the same extract and of ACh. The latter were developed with iodine, and the area of the main venom strip corresponding to these in R_F value and in colour reaction was eluted with 50% ethyl alcohol. The eluate was reduced in volume *in vacuo* and applied as single spots on five separate chromatograms and run overnight in (*a*) *n*-butanol:water, (*b*) *n*-propanol:water, (*c*) *n*-butanol:acetic acid:water, (*d*) *n*-propanol:formic acid:water, and (*e*) ethyl alcohol:hydrochloric acid. Markers of choline, ACh, PrCh, and BuCh were also placed on each paper. On development with iodine the re-chromatographed venom eluate developed in each case at one distinct spot only, closely agreeing with that of the ACh marker (Fig. 3).

Parallel assay. Parallel pharmacological tests with the active eluate from a chromatogram (*n*-propanol:formic acid:water) corresponding to ACh in R_F value and iodine reaction, gave an index of discrimination which did not deviate significantly from 1·0 in tests on the isolated guinea-pig ileum, frog rectus muscle, and blood pressure of the rabbit; the hypotensive effect of the eluate was also blocked by intravenous injection of atropine (Fig. 4).

Table 2 shows the R_F values obtained for ACh, histamine and 5-HT, and indicates clearly that with the choice of appropriate solvents a ready separation of these substances can be obtained.

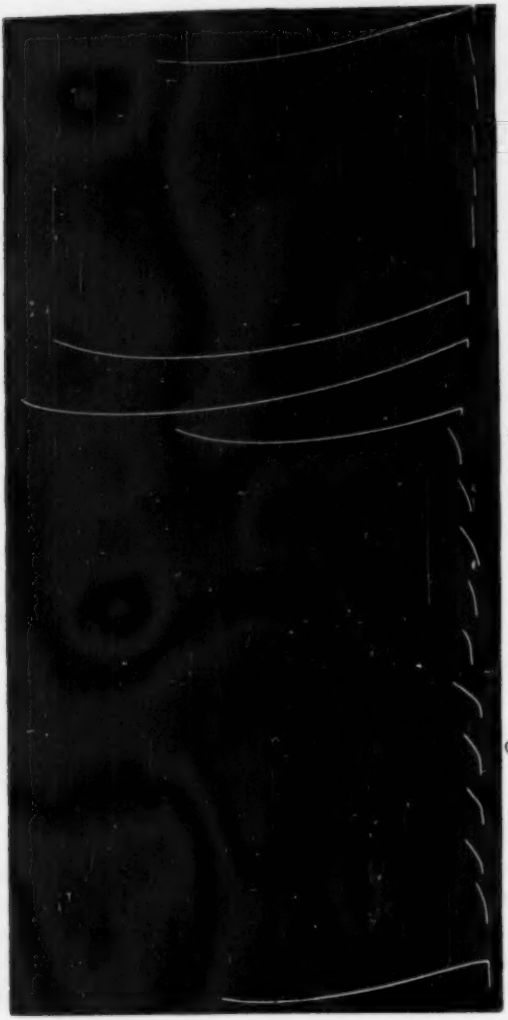

Fig. 2. Contractions of isolated frog rectus muscle (eserine 5×10^{-6} g/ml.; acetone 1/4000 (v/v)) to eluates from a chromatogram (*n*-propanol: formic acid: water) of alcohol extract of hornet venom sac. There is only a single band of activity which coincided with that of the ACh marker.

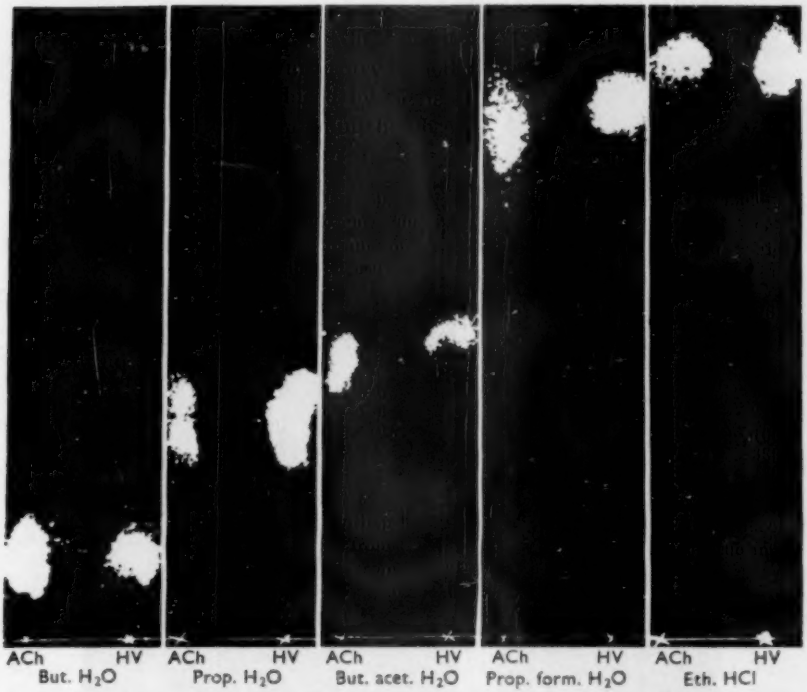

Fig. 3. Chromatographic identification of ACh in hornet venom. The ACh-like substance on a chromatogram (*n*-propanol: formic acid: water) of alcohol extract of hornet venom was eluted and rechromatographed in five different solvents. In each solvent iodine development (above) and eluted pharmacological activity corresponded to that of ACh in R_F value.

Histamine

Effect of extracts on isolated guinea-pig ileum. Acidified saline and ethanol extracts of venom sacs were assayed for histamine on the isolated ileum as for ACh, but in the presence of atropine (10^{-8} g/ml.). The activity assayed was then completely abolished by mepyramine (10^{-8} g/ml.). The histamine concentration was always less than that of ACh, varying from 50 to 80 % of that of ACh (Table 1).

Chromatography. Chromatograms in all solvents regularly yielded an active eluate which corresponded to histamine in R_F value, gave a similar Pauly reaction and caused a contraction of the guinea-pig ileum which was abolished by mepyramine.

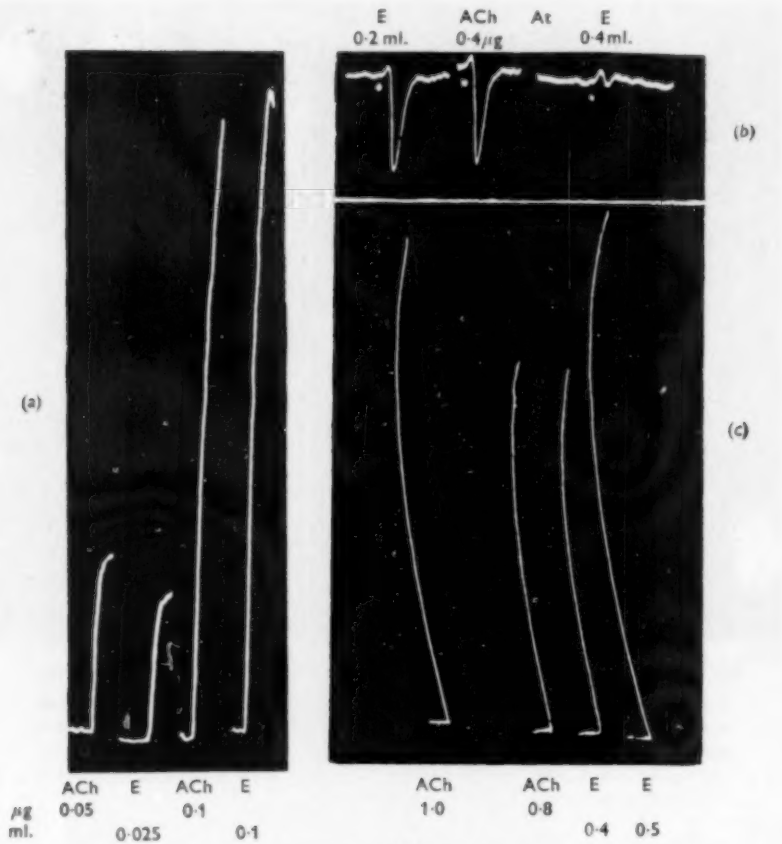

Fig. 4. Quantitative parallel activity of ACh and eluate from chromatogram (*n*-propanol:formic acid:water) of alcoholic extract of hornet venom sac. The eluate corresponded to ACh in colour reactions and in R_F value. E, eluate; At, atropine, 0·2 mg/kg. (*a*) guinea-pig ileum; (*b*) rabbit blood pressure; (*c*) frog rectus muscle.

5-Hydroxytryptamine

It was not possible to assay 5-HT in extracts of venom directly on the guinea-pig ileum because of the high concentrations of the other active substances. Even in the presence of atropine and mepyramine, when increased amounts of venom were tested the high concentrations of ACh and histamine overcame the drug antagonism. Similar difficulties developed with other test preparations. For quantitative assay and more

definite identification of 5-HT it was necessary, therefore, to use chromatography to separate it from the other substances.

Alcoholic extracts of 1–3 glands were divided equally, applied to the paper as several separate spots, and chromatographed in *n*-propanol: formic acid: water. The chromatogram showed a well defined area with the same R_F value as the 5-HT marker; it gave a red Pauly reaction, a bluish-grey colour with Ehrlich's reagent, a positive ninhydrin and ninhydrin-acetic-acid reaction—the latter showing green fluorescence under ultraviolet light and a golden-yellow colour with Shepherd's reagent. Like 5-HT the eluate from this area contracted the guinea-pig ileum (Fig. 1)

TABLE 2. R_F values of ACh, histamine, 5-HT and hornet kinin in different solvents

Solvent	ACh	Histamine	5-HT	Kinin (in ethanol extract)	Kinin (in ethanol insoluble residue)
*iso*Propanol: ammonia: water (20: 1: 1)	—	0·38	0·45	—	—
n-Butanol: ethanol: acetic acid: water (8: 2: 1: 3)	—	0·21	0·57	—	—
n-Propanol: formic acid: water (8: 1: 1)	0·64	0·16	0·40	0·40	—
n-Butanol: acetic acid: water (4: 1: 5)	0·37	—	0·40	0·34	0·35
*iso*Propanol: 0·1 N-HC (7: 3)	0·85	—	0·55	0·61	—
Ethanol: 0·2 N-HCl (1: 1)	0·63	—	0·56	0·80	0·78
n-Butanol: water (9: 1)	0·10	—	—	—	—
n-Propanol: water (9: 1)	0·26	—	—	—	—

and the isolated rat uterus, but its activity on the rat uterus was reduced by only about 50% by LSD. This suggested the presence of an active contaminant with the same R_F value as 5-HT in this solvent. This was confirmed when the LSD-resistant activity of the eluate was completely inactivated by incubation with chymotrypsin (25 μg/ml.) for 10 min (Fig. 5). The separation of the two components was then effected by rechromatographing the eluate in ethanol: HCl, the chymotrypsin-resistant material moving faster than the LSD-sensitive one (Fig. 5). Now only the latter eluate, like 5-HT, reacted with Pauly's reagent and was antagonized completely by LSD.

The concentration of 5-HT in venom was assayed on the rat uterus, using eluates (from *n*-propanol: formic acid: water chromatograms) which corresponded to 5-HT in R_F value and in Pauly reaction, after they had been incubated with chymotrypsin (25 μg/ml.) (Table 1). The concentrations of 5-HT were of the same order as those of histamine (Table 1).

Kinin

The substance contracting the rat uterus and contaminating the 5-HT area on chromatograms of venom, though inactivated rapidly by chymotrypsin (25 μg/ml.) (Fig. 5), was unaffected by trypsin. It thus differed

from wasp kinin which is inactivated by trypsin (Schachter & Thain, 1954), but resembled kallidin or bradykinin (Holdstock *et al.* 1957). It was therefore isolated further, freed from 5-HT, and its properties studied in more detail.

The area on venom chromatograms (*n*-propanol:formic acid:water) which included this substance and the associated 5-HT was eluted and re-chromatographed in several solvents. On testing the eluates re-chromatographed in *n*-butanol:acetic acid:water, activity was found in the area

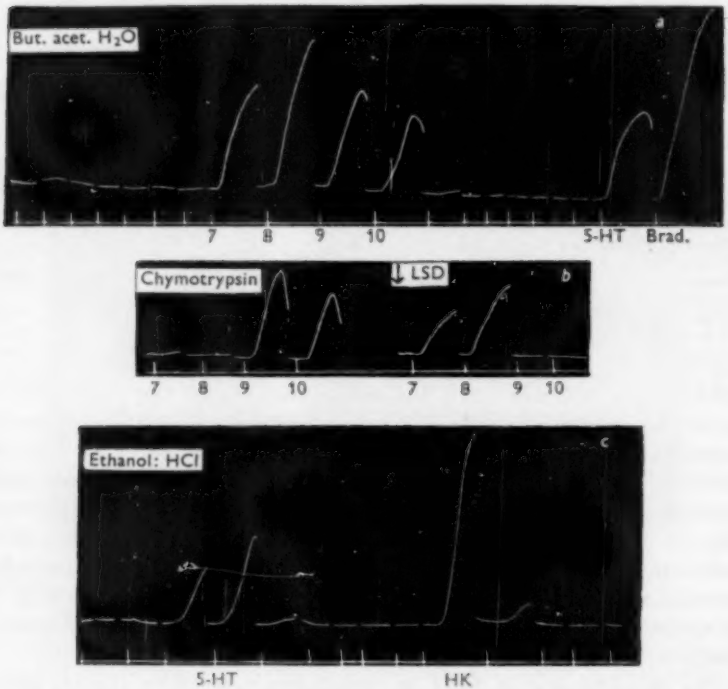

Fig. 5. Separation of 5-HT and kinin (HK) in hornet venom by paper chromatography. Contractions of isolated rat uterus (26–28° C) to chromatogram eluates. (*a*) The eluate corresponding to 5-HT in R_F value and colour reactions on a chromatogram (*n*-propanol:formic acid:water) was eluted and rechromatographed in butanol:acetic acid:water. The eluates of strips 7, 8, 9 and 10 (see Methods) were active, and the types of contraction suggested the presence of two substances inadequately separated. (*b*) The incubation of strips 7–10 with chymotrypsin (20 μg/ml., 10 min) inactivated 7 and 8 only; addition of LSD (10^{-7} g/ml.) to muscle bath antagonizes 9 and 10 only. (*c*) *Rechromatographed* pooled eluates (7, 8, 9 and 10) in ethanol: HCl; the two activities are successfully separated, the slower-moving compound being identical with 5-HT and the other with the kinin (HK).

corresponding to that reacting with Pauly's reagent and with the 5-HT marker, and it was completely antagonized by LSD (10^{-7} g/ml.). An area just behind this one on the chromatogram, but continuous with it, did not develop with Pauly's reagent and was unaffected by LSD, but was rapidly inactivated when incubated with chymotrypsin (Fig. 5). It was apparent therefore that re-chromatographing in this solvent effected a slight but still inadequate separation of 5-HT and the apparent peptides. A similarly inadequate separation was obtained with chromatograms re-run in *iso*-propanol:hydrochloric acid; in this instance 5-HT moved slightly ahead of the other activity.

A successful separation was, however, effected on re-chromatographing in ethanol:HCl. In this instance 5-HT was recovered as a sharp band with an R_F value of 0·56, whereas the LSD-resistant, but chymotrypsin-resistant material, was recovered much higher on the paper, with an R_F value of 0·80 (Fig. 5). The latter was now classified as a kinin since it also produced a slow contraction of the guinea-pig ileum, relaxation of the rat duodenum and a marked lowering of the arterial blood pressure of the rabbit. The amount of venom available did not permit precise parallel assays of this eluate with bradykinin but it was found to be relatively one tenth as active as bradykinin on the guinea-pig ileum.

Pharmacological tests of the alcohol-insoluble residue of venom sacs showed it was free from, or contained only traces of, ACh, histamine or 5-HT. It did, however, still possess much activity when tested on the rat uterus. This was unaffected by LSD, atropine or mepyramine, or by incubation with trypsin, but was rapidly inactivated by chymotrypsin (Fig. 6). The substance was studied in more detail, since the absence of other active substances in the residue permitted a simple and direct analysis. The insoluble residue when assayed against crude bradykinin on the rat uterus contained at least 350 μg bradykinin/g in terms of the pure synthetic substance. (Since, as shown below, the kinin in the alcohol extract is identical with that in the residue, the amount present in the venom is much greater than this.) It also contracted the guinea-pig ileum, relaxed the rat duodenum, and lowered the arterial blood pressure of the rabbit. It behaved as a single substance when chromatographed in *n*-butanol:acetic acid (Fig. 6) and in ethanol:HCl solvents, and had the same R_F values as the kinin in the alcohol extract. Also, a mixture of the two could not be separated by paper chromatography. It seems very likely, therefore, that these two kinins are the same substance, which is partially soluble in 95% ethyl alcohol when extracted from venom sacs.

Quantitative parallel assay of this kinin against bradykinin showed a very close similarity, but, like the alcohol-soluble kinin, it was relatively only approximately one tenth as active as bradykinin on the guinea-pig

ileum. Despite the similar behaviour of hornet kinin and oxytocin on the rat uterus there were great quantitative discrepancies in their relative activities on this and other preparations (Table 3). Hornet kinin, unlike wasp kinin, was not inactivated by trypsin (Fig. 6); mixtures of the two

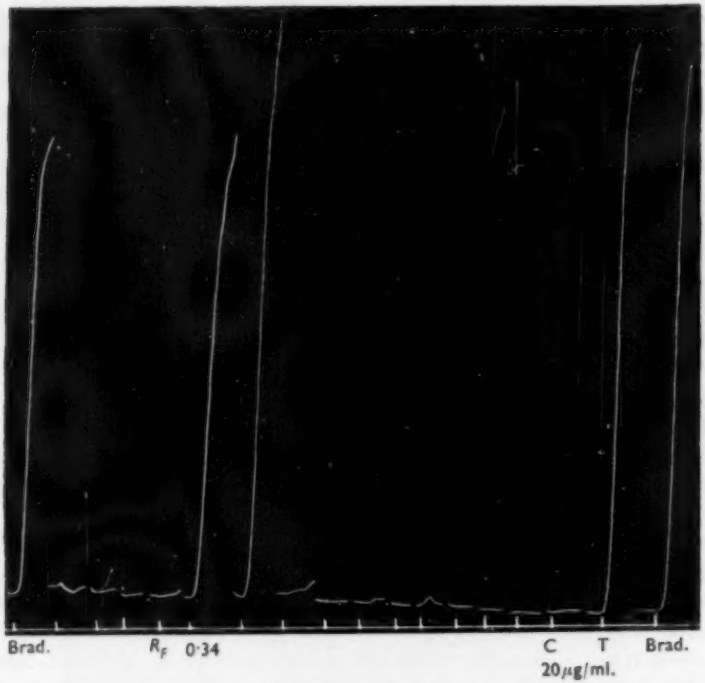

Fig. 6. Paper chromatography of hornet kinin (in alcohol-insoluble residue of venom sac) and its inactivation by chymotrypsin but not by trypsin. Contractions of rat uterus (28° C; atropine and LSD, 10^{-6} and 10^{-7} g/ml.) to eluates from chromatogram (*n*-butanol:acetic acid:water). C and T are incubated mixtures (10 min) of active eluate (R_F, 0·34) with chymotrypsin and trypsin respectively; Brad. = bradykinin, 2 u.

were also readily separated by paper chromatography in *n*-butanol:acetic acid, in which, as shown by Holdstock *et al.* (1957), only wasp kinin remained at the origin (Fig. 7). There was no evidence of wasp kinin in hornet venom since not a trace of activity was found at the origin of chromatograms in this solvent.

The similar pharmacological actions of hornet kinin and bradykinin are shown in Fig. 8; the former is relatively less active on the guinea-pig ileum.

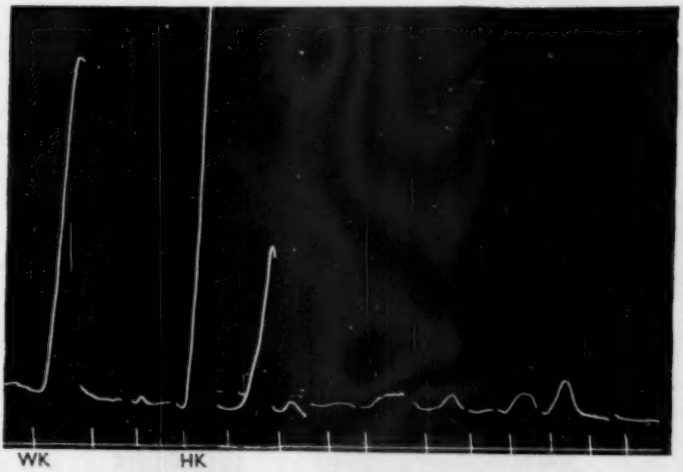

Fig. 7. Separation of wasp kinin (WK) and hornet kinin (HK) by paper chromatography (*n*-butanol:acetic acid:water). Contractions of rat uterus (28° C, atropine 10^{-6} g/ml. and LSD 10^{-7} g/ml.) to eluates from a chromatogram of a mixture of wasp and hornet kinin. One activity appears at R_F value 0 and is wasp kinin; the other at 0·34 and is hornet kinin.

Table 3. Bradykinin standard and oxytocin (Syntocinon, Sandoz) equiactive with 1 mg hornet kinin

	Bradykinin (μg)	Oxytocin (m-u.)
Guinea-pig ileum	125	—
Rat uterus	1600	800
Rat duodenum	700	23,000
Rabbit blood pressure	1600	> 85,000 (pressor)
Index of discrimination	12·8	> 100

DISCUSSION

Our experiments demonstrate that hornet venom contains four substances which are known to be among the most pharmacologically active agents present in animal tissues. There is little doubt that these substances in such concentrations, are effective in defence reactions and contribute to the severity of toxic reactions in man. The likelihood that enzymes and other proteins which add to the toxicity are present in the venom is, however, not excluded.

Hornet venom differs in composition from wasp venom in that it contains ACh and also a kinin which differs from that of the wasp. This confirms previous observations on the apparently erratic distribution of

such substances in different species of insects (Bisset *et al.* 1960) and coelenterates (Mathias, Ross & Schachter, 1960).

The presence of ACh in concentrations up to 50 mg/g in dry hornet venom sacs and of a substance resembling DMAC, in concentrations of 1–2 mg/g, in the prothoracic (defensive) gland of the Garden Tiger Moth (*A. Caja*) (Bisset *et al.* 1960), suggests that ACh and other choline esters

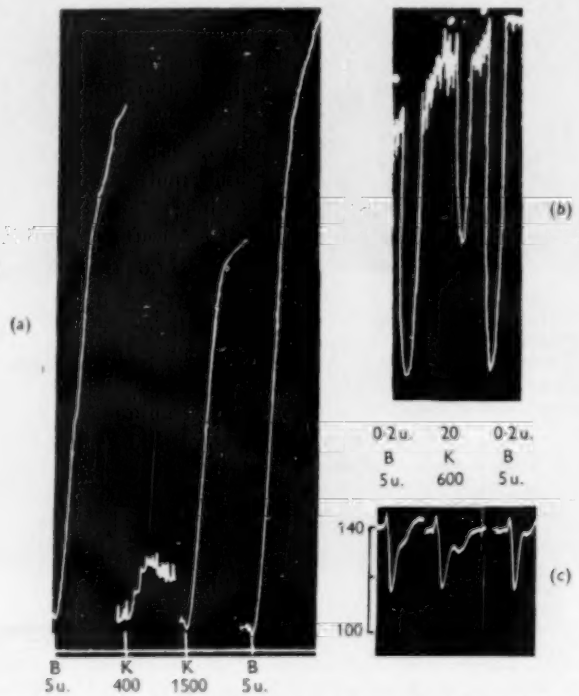

Fig. 8. Similar pharmacological actions of bradykinin and hornet kinin. (*a*) Guinea-pig ileum; (*b*) rat duodenum; (*c*) rabbit arterial B.P. B, bradykinin (units); K, hornet kinin (μg).

may be widespread in insects. The recent demonstration that the hypertrophied silk gland of the caterpillar (and cocoon) of *A. caja* and the abdominal tissue of the adult moth, contain large amounts of ACh (Morley & Schachter, 1961; Gill, Parsons & Paton, 1961) supports this view. It is possible that not only ACh, but also other choline esters, may have functions in nature other than the established one as a neuro-transmitter substance. Evidence suggesting a non-nervous metabolism of ACh in mammalian intestine has indeed been presented by Feldberg & Lin (1950).

The significance of ACh in a non-innervated organ such as human placenta (Chang & Gaddum, 1933) or of PrCh in ox spleen (Banister *et al.* 1953) has never been explained. The existence in nature of active choline esters other than ACh also underlines the need for specific identification of substances in tissues which show the general biological properties of ACh.

It has recently been shown that whole heads of various insects (flies, beetles and cockroaches) contain a substance resembling ACh in concentrations of 10–40 μg/g (Lewis & Smallman, 1956). It cannot be assumed in such experiments that the ACh is present only in the nervous tissue. The present experiments and those of Morley & Schachter (1961) demonstrate that ACh may be present in high concentrations in non-nervous tissue, and emphasizes the need for specific location of this substance.

The presence of a new kinin in hornet venom, closely resembling, but different from bradykinin (or kallidin) and wasp kinin, demonstrates that this type of pharmacologically active molecule, like choline esters and catecholamines, exists with some variations in animal tissues. It still remains to be seen whether these kinins will also be of physiological importance.

SUMMARY

1. ACh, 5-HT and histamine were identified in hornet venom sacs by parallel bioassay, paper chromatography and other chemical tests.

2. The ACh concentrations varied from 18–50 mg/g dry venom sac (mean 37·8); 5-HT and histamine were present in approximately one half the concentration of ACh.

3. A new kinin was demonstrated in hornet venom. This differs from wasp kinin in being resistant to inactivation by trypsin, in its behaviour on paper chromatograms, and in its relatively weaker action on the isolated guinea-pig ileum. It resembles bradykinin except that it is approximately one tenth relatively as active on the guinea-pig ileum as on other preparations.

4. The possible significance of ACh and other choline esters in nature is discussed.

We wish to thank Mr R. L. Ford, F.R.E.S., for providing the hornets. Part of the expenses of this work was met by a grant from Parke, Davis and Co. Ltd, which is gratefully acknowledged.

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J. Physiol. (1961), **159**, *pp.* 183–190
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THE EFFECTS OF CASTRATION ON THE VASCULAR RESPONSES OF MALE RATS TO POSTERIOR PITUITARY HORMONES

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Since the early work of Byrom (1938) it has been known that the vascular responses of the female rat to vasopressin may be modified by the concentrations of ovarian hormone in the body. Lloyd (1959*a*, *b*) has further demonstrated the changes in the vascular actions of vasopressin and oxytocin which are produced by oophorectomy, by treatment with oestrogens or progesterone, or which occur during the reproductive cycle and pregnancy. As a counterpart to this work on female rats, the present experiments are the first of a series of investigations undertaken to determine whether testicular hormones exert any influence on the vascular responses of the male rat. This paper reports the effects of orchidectomy. Since the changes in the vascular responses to oxytocin and vasopressin produced by orchidectomy differed from those which followed oophorectomy, an attempt was made to determine the nature of the differences.

METHODS

All experiments were performed on male rats weighing between 175 and 225 g. Where castration was carried out, aseptically under ether anaesthesia, the testes and seminal vesicles were both removed. Further groups of animals were sham-operated, or subjected to unilateral removal of a testis. Subsequent observations were made under anaesthesia with intraperitoneal sodium pentobarbitone (5 mg/100 g body weight). Two experimental methods were used, either singly or together; in the first the arterial blood pressure was recorded on a kymograph from the cannulated carotid artery, and in the second the blood vessels of the mesoappendix were directly observed (Chambers & Zweifach, 1944). This method was used only in relation to oxytocin on occasions when it had no demonstrable effect on the blood pressure. All injections of drugs were made into a cannulated femoral vein, in a total volume of 0·3 ml. of 0·9 % NaCl solution. The vasopressin used was Parke, Davis's Pitressin, and the oxytocin was the synthetic preparation Syntocinon (Sandoz).

RESULTS

Normal rats

The responses of seven normal male rats were studied to provide a control series for comparison with the castrated animals. In six of these the dose of vasopressin required to produce a 10 mm Hg rise in blood pressure

was between 0·8 and 1·2 m-u. In the other animal only 0·4 m-u. was required to induce this same rise in pressure; the reason for this exception was not apparent. In all seven rats oxytocin, in doses of 10–100 m-u., had no effect on the blood pressure, but caused dilatation of the mesoappendix vessels. The least intravenous dose of oxytocin required to prevent constriction by a topically applied threshold dose of adrenaline was in all cases between 50 and 75 m-u. Below this dose there was no effect on the mesoappendix vessels.

Castrated rats

Twenty-eight male rats were castrated, and the vascular responses to vasopressin and oxytocin were tested at varying intervals after operation. For the first 4 days after removal of the testes there was no change in the dose of vasopressin required to produce a 10 mm Hg rise in blood pressure; from the fifth to the tenth day inclusive the dose needed to elicit the same

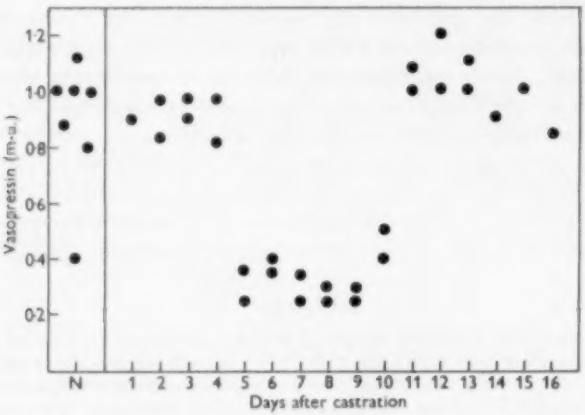

Fig. 1. The intravenous dose of vasopressin required to increase the blood pressure of the rat by 10 mm Hg before (N) and after orchidectomy.

rise in blood pressure was only 0·25–0·4 m-u.; while from the eleventh to the sixteenth days inclusive the dose required was again 0·8–1·2 m-u. (Fig. 1). Apart from this temporary increase in sensitivity to the pressor action of vasopressin other changes in the type of response were apparent. In all the rats studied between the sixth and tenth post-operative days (i.e. during the period when the sensitivity to vasopressin was increased) there was an initial depressor component to the response (Fig. 2B). This was not seen after the tenth day, by which time the sensitivity to vasopressin had returned to normal. A further change seen was in the duration of the pressor response to vasopressin. Whereas in the normal male, and for the

first 4 days after castration, the standard 10 mm Hg elevation of the blood pressure was short-lasting, the pressure being raised only for 3–5 min, all the castrated rats from the fifth post-operative day onwards showed a prolonged response (Fig. 2), lasting for some 15 min (and in one case for 30 min). This prolongation of the pressor response did not later revert to normal, but persisted throughout the period of observation. The changes in the responses to vasopressin after castration are summarized in Table 1.

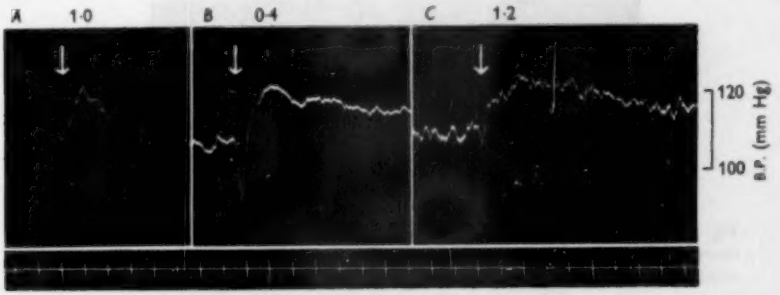

Fig. 2. The effect of vasopressin on the blood pressure of the male rat (*A*) 3, (*B*) 6, and (*C*) 12 days after castration. Numbers show doses in m-u. injected intravenously at arrows. Time marker 30 sec.

TABLE 1. The effect of vasopressin on blood pressure, and of oxytocin on blood pressure and mesoappendix vessels, before and after castration in the male rat.

Days after castration	Total no. of rats	Vasopressin	Oxytocin
Normal	7	Pressor; 10 mm Hg rise with 0·8–1·2 m-u.	No effect on B.P. Vessels dilated with 50 m-u. or more
1–4	7	Pressor; 10 mm Hg rise with 0·8–1·2 m-u.	No effect on B.P. Vessels dilated with 50 m-u. or more
5–10	12	Transient depressor then prolonged pressor response; 10 mm Hg rise with 0·25–0·4 m-u.	Depressor response with 25 m-u. or more
11–16	9	Prolonged pressor response; 10 mm Hg rise with 0·8–1·2 m-u.	No effect on B.P. Vessels dilated with 50 m-u. or more

Changes were also seen in the response to oxytocin after castration, and these followed a similar time course to the changes in response to vasopressin. For the first 4 days after orchidectomy oxytocin had no effect on the blood pressure, and the same average dose (60 m-u.) as in the normal male was required to dilate the mesoappendix vessels. From the fifth or sixth to the tenth days after castration oxytocin was able to depress the blood pressure, as well as being dilator to the mesenteric vessels, and only 25 m-u. was required to produce the depressor effect. After the tenth day a dose of 50 m-u. or more of oxytocin was again needed

to cause dilatation, as in normal rats, and this dose was without effect on the blood pressure (Fig. 3 and Table 1).

Eight observations were made serially on sham-operated male rats over a 14-day post-operative period. In all cases the responses of the sham-operated controls were indistinguishable from those of normal rats. There was no increase in sensitivity to vasopressin, and no depressor response to either oxytocin or vasopressin was seen.

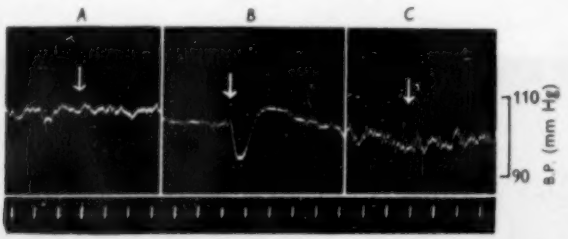

Fig. 3. The effect of 50 m-u. oxytocin, injected intravenously at arrows, on the blood pressure of the male rat (*A*) 3, (*B*) 6, and (*C*) 12 days after castration. Time marker 30 sec.

Unilateral removal of one testis was carried out in three further rats. Two of these failed to show any change from the normal in their responses to vasopressin and oxytocin at times when the changes observed after bilateral orchidectomy were maximal, i.e. between days 5 and 10 after operation. In the third, which was studied on the seventh day after operation, there was a slight initial depressor response to vasopressin, but otherwise the responses were normal.

The effects of pithing and blockade of the autonomic nervous system in castrated male rats

The unexpected appearance of a preliminary depressor response to vasopressin after castration suggested the need for a closer study of the factors underlying the constriction and dilatation caused by the posterior-lobe hormones. Since Lloyd & Pickford (1961) have shown that surgical or chemical interruption of parts of the sympathetic nervous system may alter the vascular responses of rats to these hormones, similar procedures were used in the castrated males.

A further nineteen castrated rats were studied between the sixth and tenth post-operative days, and an adrenergic blocking agent (dihydro-ergotamine, 5 rats; dibenamine, 3 rats), or a ganglionic blocking agent (tetraethylammonium iodide, 3 rats; hexamethonium, 3 rats) or atropine (3 rats), was administered during the course of the observations (doses in Table 2). The remaining two rats were pithed. After any of these pro-

cedures except the administration of atropine, the depressor response to oxytocin and the initial depressor response to vasopressin were abolished (Fig. 4). The pressor component of the effect of vasopressin was potentiated by ganglion block, but reduced in two cases after dibenamine, and once after dihydroergotamine. Atropine did not affect the responses in any way. These results are summarized in Table 2.

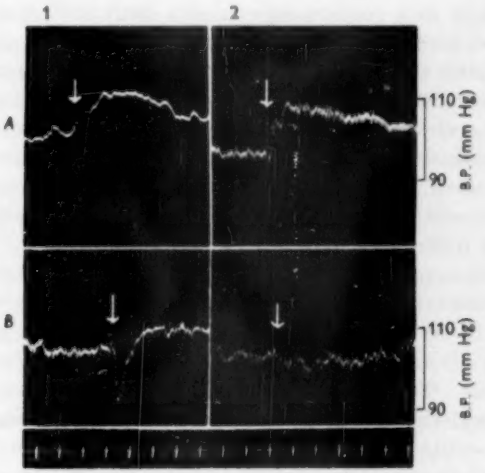

Fig. 4. The effect of (A) 0·4 m-u. vasopressin and (B) 50 m-u. oxytocin on the blood pressure of the male rat 7 days after castration; (1) before, and (2) after the intravenous administration of 0·1 mg. dihydroergotamine. Time marker 30 sec.

TABLE 2. The effect of autonomic blockade and of pithing on the responses to vasopressin and oxytocin in the castrated male rat.

Procedure	No. of rats	Vasopressin: Pressor response			Vasopressin: Depressor response		Oxytocin Depressor response	
		−	un.	+	un.	ab.	un.	ab.
Dihydroergotamine 0·1 mg	5	1	4	.	.	5	.	5
Dibenamine 0·2 mg	3	2	3	.	.	3	.	3
Tetraethylammonium 2 mg	3	.	.	3	.	3	.	3
Hexamethonium 1 mg	3	.	.	3	.	3	.	3
Atropine 0·1 mg	3	.	3	.	3	.	3	.
Pithed	2	.	.	2	.	2	.	2

− reduced; un. unaffected; + potentiated; ab. abolished.

DISCUSSION

Changes in the vascular responses of female rats to posterior pituitary hormones after oophorectomy have already been described by Lloyd (1959*a*), and the present work shows that, in the male also, castration leads to a change in the responses to vasopressin and oxytocin. In normal male and dioestrous female animals the effects of these hormones were qualitatively similar, in that vasopressin caused a short-lasting elevation of the blood pressure, while oxytocin had no effect on the blood pressure, though it visibly dilated the vessels of the mesoappendix. Quantitatively the female was more sensitive than the male to either hormone, 25–30 m-u. oxytocin being required for dilatation of the mesoappendix vessels, and 0·4 m-u. vasopressin for a 10 mm Hg rise in blood pressure in the female, as compared with 50–70 m-u. oxytocin, and 0·8–1·2 m-u. vasopressin in the male. Castrated male rats, however, showed certain temporary as well as permanent differences from oophorectomized females. The temporary differences first appeared in the males on the fifth or sixth day after operation, and in the females on the third day, and lasted until the tenth or eleventh day in the males, and until the eighth day in the female animals. In castrated male rats oxytocin had a marked depressor effect, while in the oophorectomized females there was no lowering of the blood pressure after oxytocin, though the mesenteric vessels were dilated by a smaller dose than in normal dioestrous animals. In the females the pressor effect of vasopressin was markedly reduced following oophorectomy, whereas in the castrated males the sensitivity to vasopressin was increased and the rise in pressure greatly prolonged. This increased pressor response in the male rats was preceded by a transient depressor response which was never seen in the females.

The permanent difference which followed gonadectomy was that in the males the prolonged response to vasopressin persisted for the full 16 days of observation; that is, after the time when the sensitivity to the drug had returned to normal. In the female rats there was no permanent change in the response to vasopressin, and all animals observed after the eighth day following oophorectomy showed responses to vasopressin and oxytocin which were indistinguishable from those of the normal dioestrous females (Lloyd, 1959*a*). The reason for the temporary nature of the alteration of most responses is not known, though it is possible that castration may lead to increased adrenal cortical activity, which will in turn affect vascular responses. The reason for the persistence of a prolonged response to vasopressin in male rats alone is also obscure. The changes in response seen were not due to differences in the basal blood-pressure levels, since these varied little between the groups of animals.

The changes seen suggest that the vascular responses of male rats to the posterior pituitary hormones are affected by the concentration of testicular hormones in the body, just as the responses of the females are affected by the concentrations of ovarian hormones (Lloyd, 1959*a*, *b*). However, the lack of androgens and oestrogens of gonadal origin does not appear to affect the responses in the same way. The site and mechanisms of these differences are obscure, though the experiments with blocking agents suggest that the depressor responses to both vasopressin and oxytocin in the castrated male are dependent on central nervous activity, since they were in all cases abolished by pithing or the administration of sympathetic ganglionic or peripheral blocking agents, though not by atropine. It is an interesting point that, in the normal dioestrous female, similar interference with the sympathetic nervous system enhances the pressor response to vasopressin and induces a pressor response to oxytocin (Lloyd & Pickford, 1961).

Further studies, which are in progress, are needed of the effects of androgen administration to normal and castrated males before any conclusions can be drawn as to the manner in which androgens modify the vascular responses.

SUMMARY

1. The vascular responses of male rats to vasopressin and oxytocin were studied before and after castration.

2. In normal males intravenous vasopressin, in doses of 0·8–1·2 m-u., raised the blood pressure by 10 mm Hg. Intravenous oxytocin in doses of 50–100 m-u. dilated the mesenteric blood vessels, but had no effect on the blood pressure.

3. For the first 4 days following castration there was no change in the response to either hormone. From the fifth or sixth to the tenth days inclusive the sensitivity to vasopressin was increased, and the pressor response prolonged. This was preceded by a transient fall in the blood pressure. At the same time oxytocin had a depressor action. From the eleventh day onwards the effects of both hormones were normal, except that the prolonged pressor effect of vasopressin persisted.

4. The depressor responses seen with vasopressin and oxytocin between the sixth and tenth post-operative days were abolished by pithing or by sympathetic blocking agents, but not by atropine. Following these procedures the pressor response to vasopressin was sometimes increased.

5. It is concluded that the testicular hormones may affect the vascular responses to posterior pituitary hormones, and the differences between the effects of gonadectomy in male and female rats are discussed.

We wish to express our thanks to Dr Mary Pickford for her interest and advice during this work; to the United States Air Force (contract AF 61 (052)–272) for financing this research; and to the Trustees of the Gunning Medical Bursaries of the University of Edinburgh for a grant to one of us (L. H.).

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PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

OXFORD MEETING

28–29 *July* 1961

JOINT MEETING WITH MEMBERS OF THE J. S. HALDANE CENTENARY SYMPOSIUM

Introduction

By C. G. Douglas. *University Laboratory of Physiology, Oxford.*

The chemistry of respiration

By F. J. W. Roughton. *Department of Colloid Science, University of Cambridge.*

Pulmonary gas exchange

By J. H. Comroe. *Cardiovascular Research Institute, University of California Medical Center, San Francisco, Calif., U.S.A.*

Regulation of respiration

By E. Asmussen. *Universitetets Gymnastikteoretiske Laboratorium, Copenhagen, Denmark.*

Altitude

By A. Hurtado. *Instituto de Biologia Andina, Apartado* 1116, *Lima, Peru.*

Applied physiology in the Armed Forces, with special reference to diving

By G. C. C. Damant and K. W. Donald. *Thursford, Cambridge Road, East Cowes, Isle of Wight* and *Department of Medicine, University New Buildings, Edinburgh.*

Applied physiology in industry, with special reference to mining

By R. Passmore. *Department of Physiology, University New Buildings, Edinburgh.*

A possible development of J. S. Haldane's views on the relation between quantum mechanics and biology

By J. B. S. Haldane. *Indian Statistical Institute,* 203 *Barrackpore Trunk Road, Calcutta, India.*

SCIENTIFIC MEETING OF THE PHYSIOLOGICAL SOCIETY

An apparatus for the measurement of small eye movements

By G. H. Byford and H. F. Stuart. *R.A.F. Institute of Aviation Medicine, Farnborough, Hants*

A recent investigation connected with the visual sensations experienced by a subject seated on a rotating chair required the measurement of eye movements of the order of a few minutes of arc. The corneoretinal potential is for this purpose unreliable and contains electrical artifacts of biological

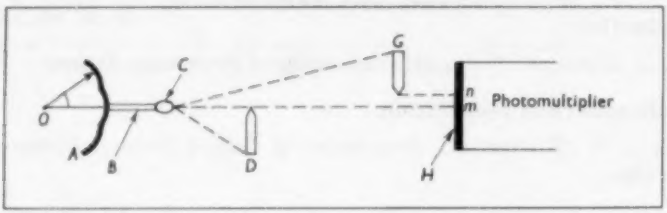

Fig. 1. Principle of the photo-electric eye movement transducer. Output from the photocell is proportional to the illuminated area m–n of the cathode, and is to a close approximation a linear function of the movement of the lamp C attached to a contact lens A. Diagram not to scale.

Fig. 2. A typical recording of steady fixation. Low gain record A, sensitivity 4 min arc/cm. High gain record B, sensitivity 45 sec arc/cm. Timing lines 0·1 sec.

origin; the highly sensitive optical techniques using a contact lens require space and mechanical rigidity, which under the present experimental conditions were not available. These disadvantages were overcome by retaining the contact lens and attaching to it an aluminium tube, at the distal

end of which a miniature electric lamp was cemented; a simple system of optical edges and a photo-electric cell (both attached to a dental bite) measured the displacement of the lamp filament and hence also the rotations of the eye.

The principle is illustrated in Fig. 1. Displacement of the lamp *C* in a plane perpendicular to that of the edge *D*, causes the shadow of this edge to move across the sensitive surface of the end-window photocell *H*. Since the edge *G* is in fact in close contact with *H*, the illuminated area of the cell cathode is to a close approximation a linear function of the displacement of *C*: the electrical output is taken via a single matching transistor to a mirror galvanometer recorder.

The bandwidth of the system is limited by resonance of the elements attached to the contact lens—approximately 800 c/s—and by the galvanometer unit. The theoretically available sensitivity is considerably in excess of that which it is possible to use in this experimental situation; 45 sec arc/cm of trace deflexion has been the maximum convenient deflexion sensitivity; noise for a 130 c/s bandwidth is then equivalent to 2 sec arc. Simultaneous photo-electric recordings and high-speed (1500 frames/sec) cine photographs of a scleral blood vessel have shown that for eye deflexions up to $\pm 10°$ from the primary visual axis, the lens and its attachments follow faithfully the rotations of the eye. Reasonable ambient illumination may be employed and normal head movements introduce no detectable artifacts. Excluding the $1\frac{1}{2}$ V cell for the transistor, the total current consumption at 500 V is 100 μA. Range and sensitivity may be changed by altering the distance *DG*, the intensity of the lamp, or the H.T. potential on the dynode chain; the recording of vertical eye movements requires the interposition of a 45° prism between *C* and *D*.

A model of a cell and its molecules

By E. H. Leach. *University Laboratory of Physiology, Oxford*

Light scattering by erythrocyte suspensions

By R. H. Kay. *University Laboratory of Physiology, Oxford*

Light transmission through blood is a complex of absorption and multiple scattering so resistant to exact calculation and sensitive to observing conditions that haemolysed samples are invariably used for accurate spectrophotometric analysis. But, in some situations, whole blood 'oximetry' may be the only usable method. It is then important to calibrate each instrument under the exact conditions of the proposed experiment (cf. Nilsson, 1960). Further investigation of the optical properties of red cells may help to evaluate possibilities and limitations of the

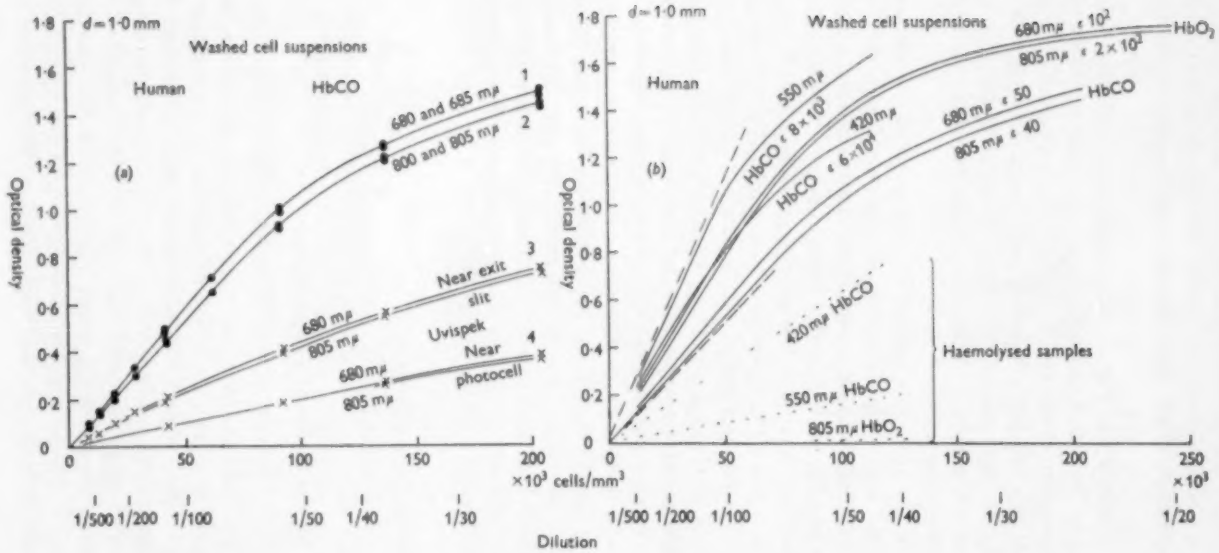

Fig. 1. (*a*) 1, 2, Extinction of red cell suspensions (HbCO) at 680 and 805 mμ. 'Uvispek 3, 4' = extinctions of the same suspensions measured in a good spectrophotometer, which are a factor two to four less depending upon how closely the sample is approximated to the photocell (within the usual measuring space), i.e. upon the acceptance solid angle for light scattered out of the strictly forward direction. With non-turbid material these differences vanish. (*b*) Extinctions at *ca.* 420 and *ca.* 550 mμ (HbCO) and at 680 and 805 mμ (HbO_2) added to curves 1 (*a*). ϵ = absorption coefficients of Hb compounds, d = 1 cm., c = 1 mM/l. Note small extinction range for suspensions with wide ϵ range.

method in new situations and assist technical development of better instruments.

There are two cases in investigating turbid media where optical density computation becomes merely difficult instead of impossible: (1) when no radiation is detected which has been scattered out of the forward direction of the incident ray, (2) when all radiation is detected no matter how great the angle to which it has been scattered. Lothian & Lewis (1956) demonstrated the apparent reversal of the intense 420 mμ haemoglobin absorption band in erythrocyte suspensions when only strictly forward transmitted light is measured; whereas when most of the scattered light is collected only a depression of intense absorptions occurs—a smoothing down of structure in the absorption spectrum. The purpose here is to extend observations of light scattering by erythrocytes to wave-lengths relevant in 'oximetry' under optically controlled conditions approaching condition (1).

Since no commercial instrument was available which could sufficiently satisfy either (1) or (2) above, a new sensitive null-point absorptiometer was designed and built in collaboration with R. and J. Beck, Ltd., London, in which the optics are suitable for meaningful measurements on turbid media, in addition to its usefulness as a general purpose absorptiometer (Kay, 1960; Beck & Beck, 1961). The instrument will be demonstrated with some measurements on washed erythrocyte suspensions (as HbO_2 and HbCO) in isotonic saline, in parallel-sided optical glass absorption cells 1 mm deep. Extinction by suspensions and absorption by haemoglobin in solution are compared by haemolysis of the sample.

Figure 1*a* summarizes measurements on (HbCO) suspensions, using narrow band interference filters at 680 and 805 mμ and a 'C' surface vacuum photocell. The optical density is almost entirely due to scattering. Single scattering persists only up to red cell concentrations *ca.* 1/50 of normal. At higher concentrations multiple scattering is involved—the criterion being deviation from Beer's Law towards weakened extinction.

Figure 1*b* adds measurements at shorter wave-lengths [Ilford 'Spectrum' filters nos. 600 (420 mμ) and 605 (550 mμ), with an 'A' surface vacuum photocell insensitive to the infra-red which these filters transmit also] and shows: (1) reversal of the 420 mμ absorption band—the ratio, optical density '420 mμ/550 mμ', being 0·8/1, whereas in the same haemolysed sample it is *ca.* 5/1; (2) that single scattering rather than absorption determines total extinction even in these intense bands; (3) that at cell concentrations greater than *ca.* 1 % of normal, multiple scattering is again dominant. The dashed lines containing all these observations (and some on HbO_2 suspensions) encompass at any concentration an optical density

range of about two to one, yet the range in haemoglobin absorption coefficients exceeds 1000:1.

In vivo, multiple scattering is unavoidable and the extinction measured is extremely sensitive to the exact geometry of the measuring instrument (cf. Lothian & Lewis and Fig. 1*a*, 'Uvispek'). Certainly, existing oximeters demand independent calibration in each experimental situation, since the very reason they measure something resembling haemoglobin absorption rather than erythrocyte scattering—a wide reception angle for multiply scattered light—implies high dependence of the instruments' indication on optical factors other than haemoglobin absorption coefficients. Extinctions which 'oximeters' record (Kay & Coxon, 1957) are much less than are measured when only forward scattered light is observed. The more closely an 'oximeter' approaches condition (2) the more useful it is likely to be.

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A polarographic method for the measurement of the oxygen consumption of the saline-perfused rat heart

By J. R. Williamson. *Department of Biochemistry, University of Oxford*

A platinum electrode rotated at a constant speed of 3000 rev/min is used to measure the concentration of oxygen in a bicarbonate-saline medium after passage through the coronary vessels of the rat heart. The perfusion apparatus is based on that used by Bleehen & Fisher (1954) and modified by the introduction of two calibration circuits, an electrode chamber, and a by-pass circuit for the heart. Fluid passes to the heart from an upper reservoir under a hydrostatic pressure of 60 cm H_2O and is returned to the reservoir by a gas lift through which 5% CO_2 in oxygen passes. The reservoir above the heart has a second outlet and fluid falls to a second gas lift, which returns it to the upper reservoir. This by-pass circuit ensures that fluid passing into the heart is fully equilibrated with the gas mixture of 95% O_2 and 5% CO_2. The two calibration circuits are simplified versions of the heart circuit and contain the same perfusion fluid. They are equilibrated with gas mixtures containing 5% CO_2 and different known mixtures of oxygen and nitrogen. The tubes and reservoirs are water-jacketed and the temperature of the water is kept constant to within $\pm 0{\cdot}1°$ C. By

means of a system of three taps, fluid either from the heart or from one of the calibration circuits is allowed to pass through the electrode chamber and on to the appropriate gas lift. The taps are specially designed to allow an uninterrupted flow of fluid round the circuits not connected to the electrode chamber. The electrode passes through a rubber bung which seals the electrode chamber and permits a limited motion of the electrode. The electrode consists of a platinum wire fused at one end to a sphere which is sealed into a glass tube. A steel sleeve is fitted over the far end of the electrode tube and seats in a self-aligning ball-race, which is set eccentrically in a circular brass disk mounted on the motor shaft of a Fracmo $\frac{1}{10}$ h.p. synchronous motor. The diameter of the circle described by the platinum sphere is 3 mm. The anode is a mercury pool in contact with saturated KCl which is connected to the perfusion fluid through a layer of agar on a sintered glass plate. A well defined plateau on the current-voltage curve is not obtained at the high speed of rotation of the electrode which is used. Instead a short region of inflexion is seen between $-0{\cdot}6$ and $-0{\cdot}8$ V, and $-0{\cdot}6$ V is normally used as the polarizing potential. The oxygen current is recorded on a chart using a Cambridge pen-recording polarograph. Electrode drift (i.e. change of the oxygen current with time at a constant oxygen concentration) is minimized by applying the potential to the electrode intermittently for periods of 10 sec each minute. The electrode is calibrated before each experiment and every 15 min during it. The change of calibration is usually within 5%. The oxygen uptake in μl./min is calculated from the product of the arteriovenous oxygen tension difference, the appropriate value of the Bunsen solubility coefficient for oxygen, and the flow rate of fluid through the heart.

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The motor innervation of mammalian muscle spindles

By I. A. Boyd. *Institute of Physiology, University of Glasgow*

The motor innervation of 125 de-afferented muscle spindles, stained with gold chloride and isolated from a number of different muscles from the hind limbs of eleven cats, was studied. Numerous normal spindles were examined in addition. In spindles from cats in which the dorsal root ganglia of segments L5–S2 were removed 3–225 days previously, two distinct types of nerve fibre and nerve ending remained. Both types of fibre disappear after the lumbar ventral roots are cut and are, therefore, somatic motor fibres; they are termed 'γ_1 fibres' and 'γ_2 fibres'. The histological evidence suggests that the γ_1 and γ_2 fibres are derived from two

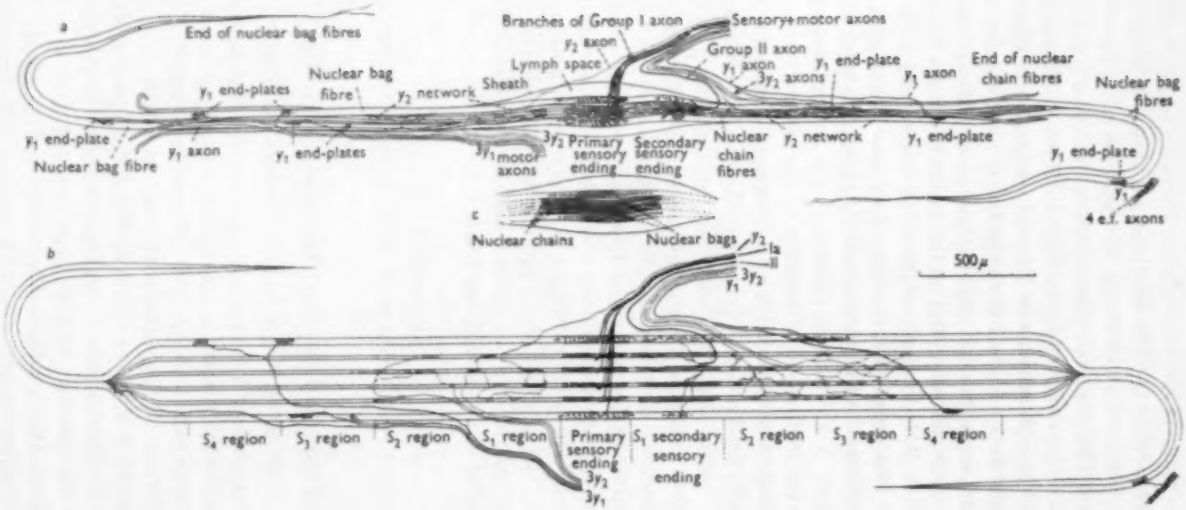

Fig. 1. A representative mammalian muscle spindle. *a*, as seen in isolated, gold-chloride stained, preparations. *b*, expanded scale diagram showing detail of spindle in *a*. *c*, nuclear region as seen in de-afferented preparations.

types of stem fibre in the muscle nerves, neither of which belongs to the α group.

The number of motor nerve fibres entering any one spindle varies from 7 to about 25. Most spindles have from 10 to 15 motor fibres of which slightly less than half are γ_1 fibres and the remainder γ_2 fibres.

Most γ_1 fibres near to the spindles have an axon diameter of 2·5–4 μ and do not branch extensively. Typically, they continue their course within the spindle to their nerve endings with little further reduction in diameter. They terminate in discrete motor end-plates always situated on nuclear bag intrafusal muscle fibres mostly in the S_3 and S_4 regions of the spindle (Fig. 1). The number of γ_1 end-plates per nuclear bag muscle fibre varies from one to six, with an average of four, and they are not unlike extrafusal motor end-plates in form. The nuclear bag muscle fibres, however, differ from extrafusal muscle fibres in a number of ways (Boyd, 1960).

Close to the spindles γ_2 fibres have an axon diameter of 1·5–2 μ, occasionally 3 μ. They undergo a sudden reduction in diameter to 1 μ or less before they enter the spindles in most cases. The γ_2 fibres branch extensively and join a communicating network of fine axons and small, elongated nerve endings termed the 'γ_2 network' situated exclusively on nuclear chain muscle fibres in most spindles. The fine terminal branches of γ_2 axons in the spindles are less than one-third of the diameter of γ_1 axons, and the γ_2 axons have been mistaken by some previous workers for sympathetic fibres. The γ_2 network lies in the S_2 and S_3 regions, and in the S_1 region close to the primary sensory nerve ending if there is no secondary sensory nerve ending in this position. If the spindle has more than one secondary sensory ending (the maximum number observed in de-efferented spindles was five; Boyd, 1959), secondary sensory terminations and γ_2 nerve endings may be superimposed on the same regions of the nuclear chain muscle fibres. The number of γ_2 endings per nuclear chain muscle fibre varies from two to a network of numerous endings which cannot be distinguished individually.

A spindle, typical of those in the muscles of the leg, is drawn to scale in Fig. 1. Full details of this investigation, and the relation of the results to those of other workers, have been submitted for publication elsewhere.

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Heat loss at parturition in ewes

By J. M. Brockway and J. D. Pullar. *Rowett Research Institute, Bucksburn, Aberdeen*

The heat loss before, during and after parturition of two 4- to 5-year-old Swaledale multiparous ewes was measured in a gradient layer direct calorimeter (Pullar, 1958). With the first animal a 24 hr measurement was made 18 days before, and thereafter the heat loss was measured continuously for the 5 days immediately preceding the birth of twin lambs. Delivery took

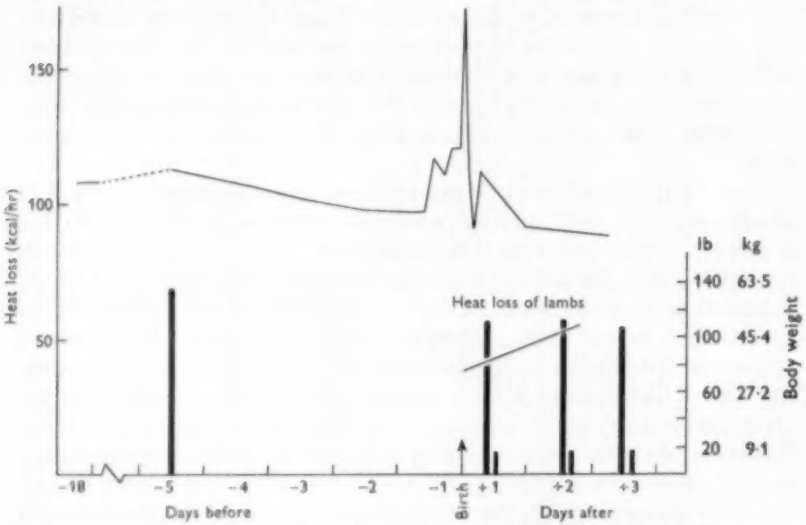

Fig. 1. Heat loss of a ewe giving birth to twin lambs. Body weight is shown by the thick vertical lines. The lower set of values on the days after birth refers to the lambs.

place in the calorimeter and measurements were continued for a further 2½ days. With the second animal, which lambed 1 week later, heat loss was measured on the 34th, 27th and 20th days before and then continuously for the 36 hr before delivery. A single lamb was born inside the calorimeter and again measurements were continued for 2½ days. The heat losses of the lambs or lamb were measured for 2 hr each day after birth; during the rest of the time measurements were made on the ewe and her offspring.

The pattern of heat loss was similar for both ewes; that for the ewe with twin lambs is shown in Fig. 1. A small increase in heat loss (from 100 to 120 kcal/hr) occurred some 12–18 hr before delivery. This increased level was irregularly maintained until just before the actual birth took place, when the total heat loss suddenly increased. During the 1½ hr period which

began with the delivery of the lambs the rate of heat loss was 170 kcal/hr for the ewe with twin lambs and 140 kcal/hr for the other ewe. These values are the total heat losses measured, minus the metabolic rate of the lambs at birth. They include the heat losses due to the cooling of the lambs, placentae and amniotic fluids. It is not possible to form an accurate estimate of these losses, which probably account for a large part of the total additional heat loss during this period. Three hours after delivery the heat loss of the ewes had fallen to about 90 kcal/hr, after which it rose to a peak of 110–120 kcal/hr and slowly declined and became stabilized again at about 90 kcal/hr. The combined heat losses from the twin lambs were about 50 kcal/hr and the loss from the single lamb was 35 kcal/hr.

These values for the energy expended during parturition may appear rather low but they agree roughly with values calculated from the oxygen consumption of women in labour quoted by Gemzell, Robbe, Stern & Ström (1957) or by Sampson, Rose & Quinn (1945) who likened the work of labour to '. . . mild to moderately heavy physical labor. . .'.

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The nature of the innervation of the nictitating membrane of the cat

By K. Hellmann* and J. W. Thompson. *Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, Examination Hall, Queen Square, London, W.C.* 1

It is generally agreed that the smooth muscle of the cat's nictitating membrane contracts in response to acetylcholine (see Thompson, 1958). However, the finding by some workers (Bacq & Fredericq, 1935; Burn & Trendelenburg, 1954; Burn & Rand, 1960) that the post-ganglionic sympathetic nerve supply to the membrane contains cholinergic as well as adrenergic fibres has not been confirmed by the results of experiments carried out on the isolated nictitating membrane (Thompson, 1960). In view of these discordant findings, the distribution of cholinesterase in the structures of the nictitating membrane was studied, since its absence would lend support to the conclusion that cholinergic fibres are absent from the sympathetic post-ganglionic nerve supply to the membrane.

* Visiting worker from Reckitt and Sons Ltd., Hull.

By means of Koelle's method for the histochemical demonstration of cholinesterase (Koelle, 1951), it was found that the smooth muscle cells of the nictitating membrane are entirely devoid of any cholinesterase activity, whilst the Harderian gland was found to contain large numbers of cholinesterase-containing nerve fibres (Fig. 1). A very few fine nerve fibres containing specific cholinesterase leave the main trunk and pass out amongst the muscle fibres, but none of them have been shown to end on muscle fibres. Larger branches of the main nerve trunk could also be seen to contain isolated cholinesterase-containing fibres amongst the rest of the fibres within the trunk.

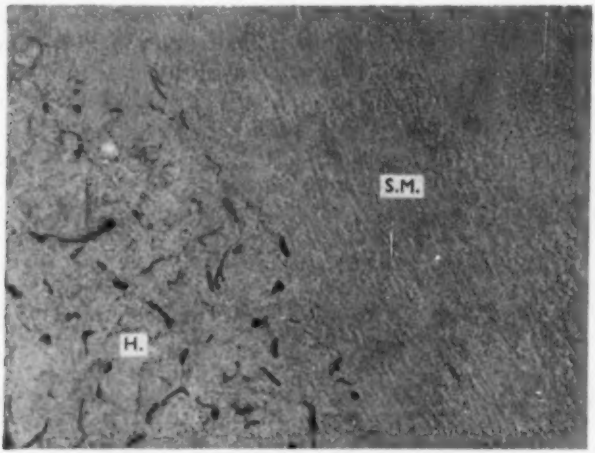

Fig. 1. Medial smooth muscle of nictitating membrane with Harderian gland. S.M., Smooth muscle showing absence of cholinesterase. H., Harderian gland intimately associated with smooth muscle and showing considerable cholinesterase activity in nerve fibres surrounding the alveoli of the gland.

The main nerve trunk passes through the muscle and breaks up into its final ramifications in the Harderian gland which is intimately associated with the muscle (Fig. 1) and the cartilage of the nictitating membrane. One or two ganglion cells were found and these stained for specific cholinesterase.

Ten days after post-ganglionic denervation of the nictitating membrane, cholinesterase activity in the Harderian gland was still present, though in reduced amounts. The absence of cholinesterase from the overwhelming majority of the nerve fibres supplying the muscle and its absence from the muscle itself lends support to the conclusion that the sympathetic innervation of the cat's nictitating membrane is entirely adrenergic. Further

support for this conclusion comes from the observation that a hemicholinium (HC3) failed to block the responses of the membrane to postganglionic nerve stimulation either *in vivo* (Wilson & Long, 1959) or *in vitro* (Gardiner & Thompson, 1961). Since the contractions of the nictitating membrane induced by acetylcholine *in vitro* are potentiated by eserine (Thompson, 1958), the present results raise the problem as to how eserine produces this effect in the absence of cholinesterase.

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An amplifier system

By P. E. K. Donaldson and D. W. Kennard. *Physiological Laboratory and Medical Psychology Laboratory, University of Cambridge*

A system of small subunits has been constructed in modular form, which can be operated in various groupings to perform different functions. The whole system is designed as a balanced differential amplifier, with a frequency range 0–10 kc/s.

The individual units perform simple functions, e.g. amplification, attenuation, etc., and require external h.t. and l.t. power supplies. Operational independence of the individual units is achieved by making both the input and output about earth potential, so that units may be used in any order. A particular feature of the design is the flexibility and ease of interconnexion between units. It is possible to check the working of modules under operating conditions without appreciable interruption of work.

Modular units. Duplication of parts and of construction increases complexity, thus the number of units must not be too great. The following types of units have been constructed:

(1) Probes. For use near tissues and organs.

(2) Amplifiers: I, low level, low noise, pentodes; II, high level, double triodes.

(3) Impedance converters. Input cathode followers, with adjustable grid current. Output cathode followers, to drive galvanometer type recorders.

(4) Attenuators.

(5) Filters. Independent high- and low-frequency filters.

(6) Calibrator; 'backing off unit'.

(7) Integrator. Miller type, with meter reading.

Practical assemblies. Complete amplifiers are assembled in frames, each housing six units with provision of power supplies for them, but larger groups can also be put together. Before use each unit is adjusted to have its output at earth potential, when the input is earthed. Thus fault finding and unit replacement is simplified.

Modular construction of instruments offers several advantages. Initially, resources need be only small for the purchase or construction of single operational units, but additions and refinements can be made as necessary. This can be of value in the provision of student apparatus, enabling fuller use of resources. Maintenance is simplified as faulty units can be replaced with little interruption of work.

In use such apparatus has proved flexible and easy to operate.

A minimal transistor stimulator ?

By W. J. Bannister. *University Laboratory of Physiology, Oxford*

This is based on an earlier circuit (Bannister & Kay, 1960) with a drastic reduction of variables to about the minimal needs of an elementary frog class and with one improvement—the substitution of an improved oscillator (T_1, T_2) based on a circuit by Bowes (1959). This is a transistor

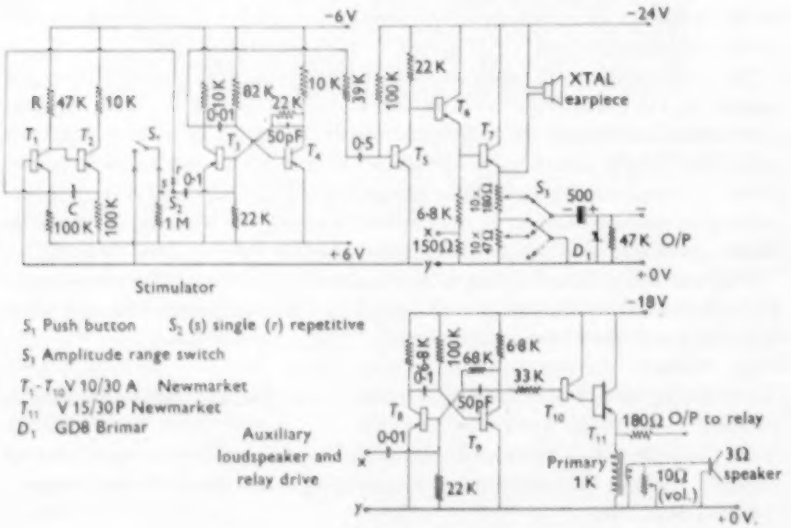

Fig. 1

analogue of the Blumlein multivibrator and both long- and short-term stability of pulse repetition frequency (p.r.f.) are good.

Specification

(1) P.r.f.—stepped control—1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50/sec.

(2) Pulse duration, 0·5 msec square.

(3) Amplitude (stepped control) two ranges, 0–4 V and 0–20 V, in steps of 0·4 and 2 V respectively, or continuous if preferred.

(4) Single pulses triggered by drum contacts, or manually by a push button.

(5) Relay stimulus marker for smoked drum driven by a brute force circuit to respond to each stimulus, whether single or repetitive.

(6) Audible monitoring by deaf-aid crystal 'earpiece'—or auxiliary loudspeaker for demonstrations.

Total battery consumption is 1·5 mA at 6 V and 2 mA at 24 V (Stimulator) and 35 mA and 18 V (Loudspeaker and Relay Drive).

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Instantaneous pulmonary blood flow measurement in man

By R. Bosman, A. J. Honour, G. de J. Lee, R. M. Marshall and F. D. Stott. *Department of the Regius Professor of Medicine, Radcliffe Infirmary, Oxford*

Pulmonary capillary blood flow may be measured in man continuously by recording the fall in pressure in a closed body plethysmograph containing the subject during a period of breath holding, after a single inspiration of 80% nitrous oxide in oxygen (Lee & Du Bois, 1955). The untrained subject finds this procedure difficult to perform, and measurement and calculation from the records is tedious. For use with the body plethysmograph, a pneumatic flowmeter has now been devised which consists essentially of a pneumatic servo system which injects or removes air from the plethysmograph and maintains a constant pressure within it. The rate of air transfer by the meter is continuously recorded. The meter has a linear response from 0 to 200 ml./sec and a uniform frequency response up to 12 c/s. In this way the rate of nitrous oxide uptake by a subject in the plethysmograph may be recorded instantaneously. From this, and simultaneous analysis of expired N_2O concentration, pulmonary capillary

blood flow may be measured easily. The blood flow is pulsatile, and from this the right ventricular stroke volume may be obtained. Comparison between this measurement and simultaneous estimations of cardiac output by the Fick oxygen method agree within $\pm 10\%$. The pattern of capillary blood flow conforms closely to that of the pulmonary arterial pressure measured simultaneously during cardiac catheterization. In the normal subject peak pulmonary blood flow follows approximately 0·2 sec after the peak pulmonary arterial pressure. The effect of pulmonary capillary blood flow on oxygen uptake can also be studied by continuously recording gas uptake when a mixture of oxygen, containing carbon dioxide at the same tension as the mixed venous blood, is breathed by the subject. Oxygen uptake is pulsatile and the pattern is similar to that of nitrous oxide uptake. By breathing nitrogen containing oxygen at the same tension as mixed venous blood, the carbon dioxide elimination throughout the cardiac cycle may also be measured. In this way the instantaneous respiratory exchange ratio of oxygen and CO_2 as blood flows through the lung capillaries may be studied.

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Orthodromic and antidromic activation of the rabbit's olfactory bulb

By C. G. PHILLIPS. T. P. S. POWELL and G. M. SHEPHERD. *University Laboratory of Physiology, Oxford*

The difference in dynamic sensitivity of primary and secondary endings of de-efferented muscle spindles

By R. J. HARVEY, J. K. S. JANSEN and P. B. C. MATTHEWS. *University Laboratory of Physiology, Oxford*

Apparatus for studying the immediate effects of X-rays on nerve and muscle

By N. ALLEN and J. G. NICHOLLS. *University Laboratory of Physiology, Oxford*

A laboratory-built van Slyke manometric blood-gas apparatus

By B. F. DICKSON and W. G. H. JOHNSON. *University Laboratory of Physiology, Oxford*

A pedal-operated fraction-collector for stop-flow analyses of urine

By W. G. H. Johnson and D. J. Ramsay. *University Laboratory of Physiology, Oxford*

Model demonstrating brain shrinkage following dehydration

By M. Bradbury and R. V. Coxon. *University Laboratory of Physiology, Oxford*

Perfusion of the cat's spleen

By A. G. H. Blakeley, C. B. Ferry, Hazel Parsons and E. H. J. Schuster. *University Laboratory of Physiology, Oxford*

Intra-arterial emboli after injury

By A. J. Honour and R. Ross Russell. *Department of the Regius Professor of Medicine, Radcliffe Infirmary, Oxford*

Growth of endothelium in fabric grafts of the aorta

By H. W. Florey and J. C. F. Poole. *Sir William Dunn School of Pathology, South Parks Road, Oxford*

A simple precision haemoglobinometer

By R. Barer and R. Underwood. *Department of Human Anatomy, Oxford*

Exchange of labelled neuromuscular blocking agent

By R. Creese, N. W. Scholes, B. E. Tilton and D. B. Taylor. *Department of Physiology, St Mary's Hospital Medical School, London, W. 2 and Department of Pharmacology, University of California at Los Angeles, U.S.A.*

The compound which has been used (iodocholinium—Creese, Taylor & Tilton, 1957) is related to decamethonium and has been labelled with ^{131}I. The exchange has been studied in isolated guinea-pig diaphragm and rabbit lumbrical muscle at 37° C.

With subparalytic doses (3 μg/ml.) an amount of radioactivity corresponding to 4–6 ml. of the external solution was concentrated in 1 g muscle in 12 hr when guinea-pig diaphragm was used. It has been found that tubocurarine markedly reduced the uptake of iodocholinium (Creese *et al.* 1957, 1959), and by this criterion the total uptake could be divided into curarine-sensitive and curarine-insensitive portions, the latter probably indicating non-specific uptake. The compound was taken up to a smaller

extent by rabbit bladder and also by rabbit tendon (0·99 ml./g after 12 hr, mean of 6 experiments), and in these cases tubocurarine had no effect.

Outward movement was studied by placing the muscle, previously loaded with the compound, in inactive flowing saline. The half-times were 226 min for guinea-pig diaphragm (mean of 14 experiments) and 374 min for rabbit lumbrical (mean of 8).

The uptake of the labelled compound was measured at different temperatures with and without tubocurarine (15 μg/ml.) so that the rate of uptake could be obtained for curarine-sensitive and curarine-insensitive portions, expressed as ml./g.min. The calculated activation energy was 7300 cal/mole for the curarine-insensitive portion and 21,000 cal/mole for the curarine-sensitive part.

The neuromuscular block produced by iodocholinium continued to increase for several hours *in vitro*, and in this it resembles depolarizing drugs and differs from tubocurarine (Jenden, 1955). The labelled compound had a slow rate of uptake and release and a high activation energy for the curarine-sensitive portion. Decamethonium labelled with ^{14}C has a high uptake as compared with labelled tubocurarine (Waser, 1960), and such differences in uptake may be related to the distinctive behaviour of these compounds.

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Transmission failure at the neuromuscular junction produced by the choline analogue, triethylaminoethanol (triethylcholine)

By W. C. Bowman and M. J. Rand. *Department of Pharmacology, School of Pharmacy, University of London, Brunswick Square, London, W.C.* 1

The acetyl ester of triethylcholine lacks the stimulating action of acetylcholine on skeletal muscle (Holton & Ing, 1949). Triethylcholine is acetylated *in vitro* by rat-brain choline acetylase almost as well as choline itself (Burgen, Burke & Desbarats-Schonbaum, 1956). If triethylcholine were acetylated in place of choline at the motor nerve endings and the resulting acetyltriethylcholine were then liberated by nerve impulses, it would be ineffective in causing muscle contraction, and transmission failure would result.

Evidence has been obtained which suggests that triethylcholine may interrupt neuromuscular transmission in this way. When injected intra-

venously into anaesthetized cats or rabbits, triethylcholine caused a slowly developing decrease in the height of contractions of the tibialis anterior muscle stimulated rapidly through its nerve, but was without effect on the contractions of the contra-lateral tibialis anterior muscle which was stimulated at a lower rate. During the paralysis of the rapidly stimulated muscle conduction along the motor nerve was unaffected and the muscle responded normally both to direct electrical stimulation and to acetylcholine injected close-arterially. In conscious chicks, cats, rabbits and dogs, injection of triethylcholine caused pronounced weakness in exercised muscles but had less effect when the animals were restrained. The failure of neuromuscular transmission produced by triethylcholine was reversed by an injection of choline.

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On the mechanism of post-tetanic hyperpolarization in myelinated nerve fibres from the frog

By R. W. STRAUB. *Albert Einstein College of Medicine, New York* 61, *N.Y., U.S.A., and Physiological Institute, University of Cologne, Germany*

In nerve fibres of small calibre a train of impulses is followed by a long-lasting period of hyperpolarization which seems to be caused by metabolic extrusion of sodium ions (Ritchie & Straub, 1957; Connelly, 1959). The way by which extrusion of sodium may raise the membrane potential is not fully understood. Qualitatively it seems possible that the hyperpolarization is caused by a decrease in the [K] outside the axon, if Na extrusion is coupled with K uptake. Connelly's experiments, however, suggest that a coupled Na-K pump at the node could not explain the post-tetanic hyperpolarization in myelinated fibres, and that uncoupled or electrogenic Na extrusion may be the main cause. Further evidence for the electrogenic mechanism was found in the present experiments.

Bundles of nerve fibres from the tibial nerve were mounted in a sucrose-gap apparatus and stimulated at intervals of 10 min for 5 sec with 200 shocks/sec. Measurements of the post-tetanic hyperpolarization and of the membrane resistance showed that for solutions with [K] of 2·5–11 mM the amplitude of the post-tetanic hyperpolarization was directly proportional to the membrane resistance. The same relation applied to the slow decrease in post-tetanic hyperpolarization and in membrane resistance observed during prolonged experiments with the preparations in Ringer's solution.

In K-free solution large post-tetanic hyperpolarizations were regularly found: their amplitude had about twice the value expected from the above relation.

Other experiments showed that the membrane potential during the post-tetanic hyperpolarization could greatly exceed the resting potential in K-free solution, sometimes by as much as 9 mV. Further, from the short-circuiting factor and the resting potential after small changes in the [K] of the bathing solution, the potential with 0 mM-K was estimated with the equation of the membrane potential maintained by a neutral pump (Hodgkin, 1957). For instance, in an experiment with a short-circuiting factor of 0·6, a depolarization of 1·8 mV was recorded when the [K] was raised from 2·5 to 3·75 mM; the post-tetanic hyperpolarization was 11·3 mV and the hyperpolarization calculated for a decrease in $[K]_0$ to 0 mM was 4·5 mV.

In some experiments the hyperpolarization recorded after stimulating for 30 sec with 50 shocks/sec was 9–10 mV. From the Na uptake during activity (Asano & Hurlbut, 1958), and the membrane potential and the intracellular ion concentrations in the resting nerve, it can be estimated that the post-tetanic increase in the intracellular [Na] would have been much too small to produce the observed hyperpolarization by coupled Na-K transport. A similar calculation suggests that an uncoupled Na pump at the node could account for the hyperpolarization, even if only half of the metabolic Na efflux were electrogenic.

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Taste functions of carp (*Cyprinus carpio* (L.))

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The dynamic responses to slow stretch of muscle spindles in the decerebrate cat

By J. K. S. Jansen* and P. B. C. Matthews. *University Laboratory of Physiology, Oxford*

Primary endings of de-efferented muscle spindles of soleus are much more sensitive to the dynamic component of slow stretch than are secondary endings (Harvey & Matthews, 1961). The present report con-

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cerns the effects of physiologically maintained 'γ bias' on the responses of these endings to slow stretch (13 mm, at 3 mm/sec).

With spinal roots virtually intact both primary and secondary endings (classified on afferent fibre conduction velocity) usually fired more rapidly throughout the stretch than when subsequently de-efferented by ventral root section or curarization. During such maintained 'γ bias' the dynamic responses of secondary endings were usually even less than after subsequent de-efferentation (dynamic responses assessed by frequency decrease on completion of dynamic phase of stretching). 'γ bias' thus mainly increased their static response.

In contrast, for primary endings 'γ bias' often increased the dynamic as well as the static response, but there was no simple relation between the changes in these two kinds of response. Two extreme effects occurred. Some 'biased' endings were much more dynamically sensitive than when subsequently de-efferented, though their response during maintained extension was only slightly increased. A few 'biased' endings, however, showed much less dynamic sensitivity than when de-efferented, while their response during maintained extension was increased. Most endings showed intermediate responses. These effects were independent of initial length and extent of stretch within physiological limits and were reflected by changes in discharge pattern as stretching began. Large dynamic responses still occurred after dorsal root section.

From these results we suggest that the static and dynamic responses of primary endings are under relatively independent control. This could occur if different portions of their nerve terminals had different relative static and dynamic sensitivities (cf. Katz, 1961), and if these portions could be influenced independently by intrafusal fibre contraction. Two kinds of intrafusal muscle fibre exist, probably with independent motor supply, permitting elaboration of this hypothesis. The nuclear chain intrafusal fibres receive almost all the terminations of secondary endings, and also substantial branches of the primary ending (Boyd, 1959, 1960, 1961). We suggest these branches contribute static, but little dynamic, sensitivity to the over-all response of the primary ending. The nuclear bag intrafusal fibres receive further branches of the primary ending; we suggest these branches contribute most of the dynamic sensitivity of the ending as well as some static sensitivity. Contraction of nuclear bag fibres probably increases the dynamic sensitivity of the ending, while contraction of nuclear chain fibres, which are in parallel with the nuclear bag fibres, might unload the latter and decrease the over-all dynamic sensitivity of the ending, while increasing its static response. This would provide a mechanism for controlling phase advance, a property of the ending important for the servo control of movement (Merton, 1951).

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The extensibility of voluntarily contracted muscles

By J. G. ROBSON. *Physiological Laboratory, University of Cambridge*

Wilkie (1949) showed that the force produced at the wrist by maximal voluntary contraction of the flexor muscles of the elbow is practically independent of the angle of the elbow for angles of about 90°. Thus it appears that when they are maximally activated these muscles are very extensible. However, Matthews (1959) found that the cat's soleus muscle is less extensible during low-frequency stimulation than it is when stimulated at a high rate, and it seemed possible that the elbow flexor muscles might also be less extensible when less than maximally active.

This possibility was investigated by determining the relation between the force produced at the wrist by voluntary flexion of the elbow, the angle at the elbow, and the mean amplitude of the electromyogram recorded from electrodes over the biceps muscle.

In agreement with Wilkie (1949) it was found that the maximum force which could be developed at the wrist was virtually independent of the angle of the elbow in the range 83°–97°. The mean amplitude of the electromyogram, however, increased by about 1 % for each degree decrease in the angle at the elbow, not only when any constant submaximal force was maintained but also when maximum effort was exerted.

This finding makes it seem improbable that the extensibility of the muscles flexing the elbow is significantly less during normal voluntary contraction than when a maximum effort is made.

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Hormonal basis for impairment in milk synthesis and milk ejection following hypothalamic lesions

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Liberation of small quantities of sympathins from the cat's adrenal gland

By E. MARLEY. *Department of Pharmacology, Royal College of Surgeons of England, and the Institute of Psychiatry, Denmark Hill, London, S.E. 5*

An adaptation of Vane's (1958) blood-bathed isolated organ technique was used. In this, arterial blood from an anaesthetized heparinized animal bathes continuously an assay tissue before being returned to the venous circulation.

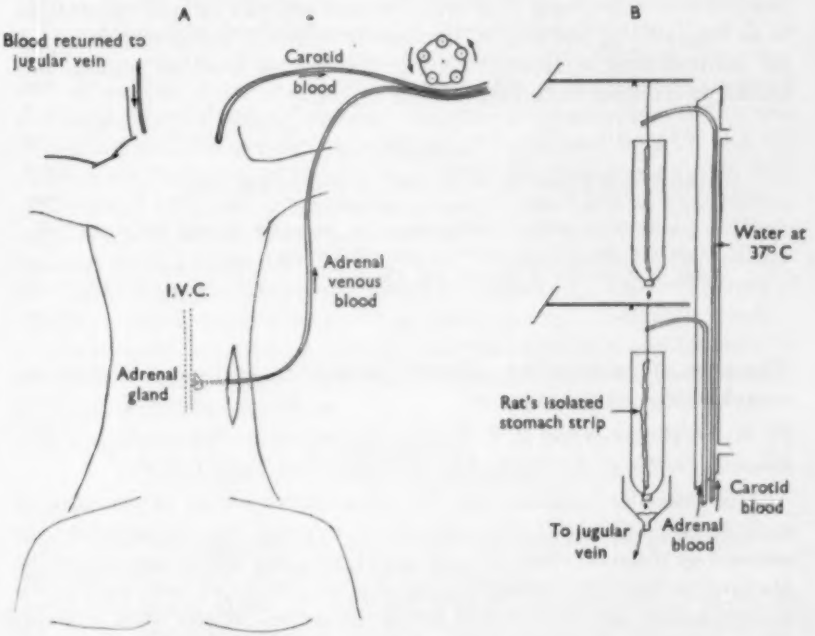

Fig. 1

Adrenal venous blood (3–5 ml./min) as well as carotid arterial blood (4–6 ml./min) was pumped from an anaesthetized cat through silicone tubing by a roller-pump (Saxby, Siddiqi & Walker, 1960). One end of the adrenal cannula was tied into the adrenolumbar vein lateral to the gland and slightly overlying it; the other end was brought out through a flank incision (Fig. 1 A). The opening of the adrenolumbar vein into the inferior vena cava was left patent; some vena caval blood mixed with adrenal blood was therefore pumped from the animal. The opposite adrenal gland was excised. The separate arterial and venous bloods were warmed in a

water-jacket at 37° C, and either mixed before superfusing a rat's isolated stomach strip (Vane, 1957) or the blood streams superfused strips in series (Fig. 1B), the upper strip receiving carotid, and the lower adrenal and carotid blood, before return to the jugular vein. The tone of the strip was recorded by an auxotonic lever (Paton, 1957) of 22:1 magnification.

Release of adrenal sympathins into the general circulation could just be detected when the greater splanchnic nerve was stimulated at 30 shocks/sec for 20 shocks or with sustained excitation at 2–4 shocks/sec (Marley & Paton, 1961). By monitoring output from the gland directly it was possible to detect release after only 2 shocks at a rate of 1/sec, equivalent to as little as 5 ng adrenaline. Sympathin release followed stimulation of the contralateral splanchnic nerves; for this at least 200 shocks was needed at minimal excitation rates of 10/sec.

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The role of calcium in adrenal medullary secretion evoked by acetylcholine or potassium

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It is generally believed that the sympathetic nerves to the adrenal medulla are cholinergic. It is not known, however, how the acetylcholine released by these nerves evokes the secretion of catecholamines. Since the transmitter function of acetylcholine at other sites in the body such as the neuromuscular junction seems to be concerned chiefly with inducing changes in the permeability of the 'receptor' membrane to common species of ions, we have studied how changes in the extracellular concentration of sodium, potassium or calcium influence the secretory response of the adrenal medulla to acetylcholine.

The experiments were done on cat's adrenal glands perfused at 24–26°C with Locke's solution or appropriate variations of it. In some experiments the adrenal gland was perfused *in vivo* through the abdominal aorta after acute sympathetic denervation, and effluent was collected from the adrenal vein. More often an adrenal gland was removed from the cat, placed in liquid paraffin, perfused through the adrenal vein in a direction the reverse of normal and effluent collected from the many severed small arteries. The

catecholamine content of the effluent was determined by bioassay with the rabbit aorta preparation (Furchgott, 1960).

The resting output of catecholamines was about 50–100 ng/min/gland (somewhat higher values were usually observed at the beginning of an experiment). When ACh (10^{-5} g/ml.) was added to the Locke's solution perfusing the gland, catecholamine output rose to about 10 μg/min/gland. Removal of sodium from the perfusion fluid (tonicity being maintained with sucrose) for 16 min did not greatly diminish the response to acetylcholine; while perfusion with potassium-free Locke's solution for a similar time increased the response to ACh by about 50%. In contrast, perfusion with calcium-free Locke's solution depressed the response to ACh to between 5 and 1% of the control response in Locke's solution. The omission of calcium from the perfusion fluid also prevented the powerful stimulant effect of excess potassium (56 mM). A quantitative relation was found between the amount of catecholamines released by ACh and the calcium concentration of the perfusion fluid: the responses to ACh with 0·5, 8·8, and 17·6 mM calcium were about 50, 150 and 200% of the control responses with 2·2 mM calcium. Excess calcium (20 mM) added to Locke's solution did not cause secretion, but after 16 min perfusion with calcium-free Locke's solution the reintroduction of calcium (2·2 mM) in the form of Locke's solution raised secretion temporarily to about 10 μg/min/gland.

These results suggest to us that the role of acetylcholine as a transmitter at the adrenal medulla may be to cause the adrenal cells to take up calcium, perhaps by depolarizing them.

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The reduction in sodium reabsorption caused by perfusion of kidneys with blood from headless donors

By T. DE LIMA, MARY F. LOCKETT and C. ROBERTS. *Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W.* 3

Cat kidneys perfused with blood from headless donor animals excreted a greater percentage of the filtered load of sodium ($t_{calc.} = 4{\cdot}2$, $n = 64$, $P = < 0{\cdot}001$) at a faster rate of urine flow ($t_{calc} = 5{\cdot}7$, $n = 64$, $P = < 0{\cdot}001$) than did those perfused with blood taken from intact or acutely adrenalectomized animals (de Lima & Lockett, 1961). This was so whether the donor animals had been decapitated under chloralose anaesthesia, or had, instead, been made spinal. Vasopressin (Pitressin; Parke, Davis: 20 μu./150 ml. blood) reduced the excessive water loss of preparations perfused with blood from headless spinal animals, but failed (10 m-u./150 ml.)

to do so in those perfused with blood from headless chloralosed donors. In neither case did vasopressin reduce the excessive rate of sodium loss. Neither did oxytocin (Pitocin; Parke, Davis: 10 m-u. or Syntocinon; Sandoz: 10 m-u./150 ml.), nor a 1:1 mixture of vasopressin and oxytocin (20 m-u./150 ml.) decrease the high proportion of the filtered sodium excreted when blood from headless donors was used. Whole posterior pituitary extract (Infundin; Burroughs Wellcome: 10 m-u./100 ml. batch 91303) did, but less effectively than saline extract of whole ox pituitary equivalent to 2 mg fresh gland.

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Chemoreceptors and shivering

By L. F. FAGAN* and JOAN C. MOTT. *Nuffield Institute for Medical Research, University of Oxford*

It is now well known that if all but the youngest new-born mammals are placed in a subneutral environmental temperature, their O_2 consumption diminishes when the pO_2 of the inspired air is reduced (Dawes, 1961), but the mechanism of this phenomenon is not fully understood. In 1958 von Euler & Söderberg observed that shivering was inhibited in lightly anaesthetized cats by the intravenous injection of small doses of lobeline as well as by hypoxia. They suggested that this might be due to the stimulation of peripheral chemoreceptors having a reflex action through hypothalamic centres. Since lobeline is known to stimulate sensory endings other than the aortic and carotid chemoreceptors we have attempted to inhibit shivering by other means. Experiments in rabbits, lightly anaesthetized with pentobarbitone, have shown that:

(1) Electrical stimulation of the central end of the carotid sinus nerve sufficient to induce over-ventilation also stopped shivering.

(2) The O_2 content of the inspired air at which shivering ceased in rabbits, breathing from a closed circuit containing soda-lime, was higher (10·2–7·5 %) when the vagi and carotid sinus nerves were intact, than after cutting these nerves (6·1–3·8 %). In these experiments, oxygen consumption always fell when shivering ceased.

(3) Shivering was readily inhibited by rebreathing air or by inhaling 8–9 % O_2 in N_2. This inhibition was diminished by denervation of the carotid bifurcations after the vagi had been cut.

* Supported by a Fellowship from the National Heart Institute, U.S. Public Health Service.

(4) Perfusion of the carotid bifurcation by a method similar to that of Joels, Neil & Hudson (1960) has resulted in a diminution of shivering when the perfusate is equilibrated with 5% CO_2 in N_2, as compared with 5% CO_2 in O_2.

(5) The reflex hyperpnoea elicited by intravenous injection of 400–1200 μg sodium cyanide accompanied by a decrease of shivering. When this hyperpnoea was abolished by denervation of the sinus areas, the inhibition of shivering caused by sodium cyanide also disappeared.

These experiments support the idea that chemoreceptor stimulation inhibits shivering.

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The development of some rhythmic activity in the electroencephalogram of the young dog

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The electroencephalogram (e.e.g.) of the new-born dog shows a somewhat irregular activity at 8–15 c/s, mixed with slower waves. This activity tends to appear in short bursts separated by periods of relative equipotentiality lasting one or more seconds. This activity is quite different from that of the adult dog. In order to follow the development of regular patterns of rhythmic activity in the e.e.g., forty-two dogs were examined at various intervals during the first 6 months of life. Small stainless-steel surgical needles (Lane's 'cleft palate' $\frac{3}{8}$ in., 9 mm.) were used as electrodes lightly inserted in the scalp over the anterior, middle and posterior third of each hemisphere. The animal was gently held or nursed on the lap of a laboratory assistant.

It was found that nearly continuous 12–15 c/s activity would appear from both the anterior and the posterior halves of the hemispheres during the course of the second week of life. Later, slower waves became predominant and between the age of 6 and 8 weeks a regular, rhythmic activity of the order of 100 μV or more was well established. This activity at about 3–5 c/s was much larger over the posterior than over the anterior half of the head. It would easily appear if vision was excluded and would disappear if vision was restored. It would disappear in response to a variety of stimuli even when the exclusion of vision persisted. This rhythmic activity would also disappear during drowsiness and sleep but

could be made to reappear with the presentation of a variety of stimuli. After the end of the third month there was a gradual diminution in the amplitude of this rhythmic activity and in the fifth and sixth months an activity at about 5–7 c/s became predominant over the posterior half of the hemispheres, mixed with faster activities. This pattern, of low amplitude, is similar to that described in adult dogs.

It appears that various phases occur in the development of rhythmic activity in the electroencephalogram of the young dog in the first six months of life. The changes in pattern of rhythmic activity appear fairly closely related to age.

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The influence of penicillin on steady cortical potentials

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Topical application of penicillin to the cerebral cortex produces intense myoclonic movements (Walker & Johnson, 1946) and e.e.g. changes typical of grand-mal seizures (Ralston, 1958). The experiments reported here were undertaken to ascertain the accompanying changes in the steady potentials of the cortex (Goldring & O'Leary, 1951).

The potential difference between two silver electrodes, one on the surface of the cortex and another on the skull of a rat, anaesthetized with pentobarbitone, was measured by a cathode follower and galvanometer system. Similar measurements were made of the potential difference between the indifferent electrode on the skull and a glass micropipette (outside diameter 1–3 μ) at varying depths below the surface of the cortex. A comparison of the results of the two measurements showed that a potential gradient was found to exist between the surface and the deeper layers of the cerebral cortex.

Topical application of penicillin dissolved in c.s.f.-Locke (pH 7·0) in concentrations of $4{\cdot}0–6{\cdot}0 \times 10^{-2}$ M produced characteristic changes in this gradient. The effect of this solution was to make the cortical surface progressively more and more negative in relation to the indifferent electrode, whereas the deeper layers, below 500 μ, did not show any significant changes in relation to the indifferent electrode. There was thus produced an increased gradient between the surface and deeper layers of the cortex. As the gradient increased, episodes of repetitive activity appeared. This repetitive activity was characterized by waves of 200–250 msec duration.

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They appeared initially at a frequency of 1/sec, which increased as the potential gradient increased. The episode terminated abruptly with a sudden large increase in negativity of the deeper layers, and a smaller increase in negativity of the surface layer. The potential gradient across the cortex thus became less. During this terminal phase the repetitive activity became more rapid, and the waves were of shorter duration. The end of the episode was marked by a sudden decrease in surface negativity and a cessation of repetitive activity while the negativity of the deeper layers decreased more slowly until the normal gradient (surface negative to the deeper layers) was re-established.

These experiments suggest that the repetitive episodic activity produced by penicillin is dependent on a depolarization of the superficial cortical structures, resulting in an increased potential gradient between surface and underlying cortex.

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Changes in cerebellar electrical activity during the development of conditioned reflexes

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Changes in cerebellar electrical activity during the development of a motor conditioned reflex were studied in non-anaesthetized, unrestrained cats, in whom platinum electrodes had been implanted over the auditory areas of the cerebral cortex and over the cerebellar vermis. Provision was also made to record the activity of subcortical structures with a tungsten wire electrode. An indifferent electrode was placed over the frontal sinus and with an oscilloscope and an e.e.g. pen recorder monopolar recordings from these positions were obtained. Once the animals were fully recovered from the operation records were obtained of the normal electrocortical activity and the responses evoked by sounds of 120 msec duration at a frequency of 1000 c/s, applied in groups of five and six, and separated by intervals of 1–2 sec. This control period lasted 6–8 days, after which every group of five or six sounds was reinforced by means of a small amount of food, and a motor conditioned response was established.

During the period of control the electrocerebellogram was characterized by low amplitude high-frequency waves (10–50 μV and 120–150/sec),

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although less frequent and higher amplitude waves could also be seen (50–80 μV, 10–40/sec). Sound stimuli applied during this period produced desynchronization of the electrocerebellogram in about 30% of the trials. As conditioning developed, the sound stimulation elicited a more and more frequent desynchronization of the slow waves until eventually it appeared in 80% of the trials. Similar modifications of the slow waves were observed in the auditory cortex and subcortical structures.

The responses evoked by the auditory stimulus and recorded from the cerebellar vermis showed also a definite change with conditioning. The responses obtained at the beginning of the control period showed marked habituation, disappearing after repetitive stimulation. However, once the conditioning procedure was established, this evoked cerebellar potential reappeared and was consistently evoked by the sound stimulus.

These results indicate that two of the characteristic changes that have been observed in different parts of the central nervous system during the development of a conditioned reflex (Beck, Doty & Kooi, 1958) can also be obtained from the cerebellar cortex.

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Functional characteristics of the anterolateral (spinothalamic) system in the cat

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The spectral sensitivity of the dark-adapted grey squirrel

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The whole of the pure cone retina of the grey squirrel (*Sciurus carolinensis leucotis*) has neural connexions which might provide acuity approaching that of the human fovea (Walls, 1942; Arden & Tansley, 1955). Electrophysiological investigations have shown that an unusual spectral sensitivity curve $\lambda_{max.}$ *ca.* 530 mμ, is in good agreement with the *in vivo* determination of the difference spectrum of the visual pigment of the retina (Weale, 1955). However, extracts of the retina yielded a pigment max. 502 mμ, and a hypothesis was advanced to reconcile these differing findings (Dartnall, 1960). A behavioural method has allowed us to determine the squirrels' absolute and relative spectral sensitivity under dark-adapted

conditions. The animals were reared from birth in the laboratory, and were trained to associate a signal light with a food reward. The threshold determinations were carried out in a modified Y-maze. The squirrel was presented with a stimulus only when it returned to a perch at the back of the maze, the floor of which contained micro-switches so that the squirrel's movements could be followed from outside the completely blacked-out box. The stimulus light was projected on to either of a pair of opal glass windows which subtended an angle of approximately 2° at the perch. The wave-length of the light was selected by interference filters (Balzer) and the intensity varied by neutral density wedges and filters. The light source was supplied by a constant voltage transformer.

The squirrels made 95% correct responses to sufficiently intense stimuli. When the intensity was reduced, the percentage of correct responses fell. Over a range of about 1 log unit, there was a drop in performance from 75 to 50% (chance) level. Threshold criterion was taken as 60% correct. A randomly chosen series of intensities and positions of stimuli was presented. One run of 100–200 choices could be recorded in an hour, and was sufficient for the determination of a threshold. The absolute sensitivity was found to approach that of the human scotopic system. This was surprising, because the squirrel has a purely diurnal habit. The spectral sensitivity curve appears to have two maxima, and is broader than could be expected if vision under these conditions were mediated by visual purple alone.

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Some properties of intercostal muscle in the cat

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Intercostal muscle has been likened to and contrasted with other muscles but there is no direct evidence available concerning its characteristics.

The parameters specified by Buller, Eccles & Eccles (1960) characterizing a muscle as fast or slow have been studied in slips of cat intercostal muscle, of which the blood supply was preserved. Isometric twitch and tetanic responses to indirect stimulation have been recorded from slips of muscle in the upper seven spaces on both sides of the body. A flat spring myograph and mechano-electrical transducer valve,

RCA 5734, were used. Internal intercostal slips were taken parasternally, where the external intercostal is not present, and the transversus thoracis was dissected from their underside. Recordings were also made from the two intercostals and the transversus thoracis together, underlying serratus anterior. Contractions of soleus and tibialis anterior in response to nerve stimulation were also recorded in some animals. The temperature of the muscles was maintained at 37–38° C.

TABLE 1

	Single contractions						Tetani			
Muscle	No. of cats	No. of observations	Time to peak (msec)	S.D.	Time to half relaxation (msec)	S.D.	No. of cats	No. of observations	Interval between stimuli at half max. tetanus (msec)	S.D.
Parasternal intercostal	17	126	32·1	±3·9	32·4	±7·8	17	17	35·4	±4·8
External and internal intercostal with transverse thoracis	14	138	34·1	±6·6	35·4	±8·2	7	7	36·3	±5·0
Soleus	2	23	86·0	±4·3	90·4	±10·4	4	4	96·0	±6·0
Tibialis anterior	2	12	27·3	±2·0	27·0	±2·0	3	3	41·0	±1·6

The results for single twitches and tetani summarized in Table 1 show that the intercostals resemble the tibialis anterior rather than soleus.

Additional evidence for this similarity was shown by the effects of D-tubocurarine chloride and decamethonium (cf. Paton & Zaimis, 1951). Curare was less effective and decamethonium more effective on the intercostals and on tibialis anterior than on soleus.

These results suggest that the intercostal musculature in the upper seven spaces in the cat is fast in character, though not as fast as tibialis anterior and some other muscles.

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The effect of subarachnoid injections of Cu^{2+} in pigeons

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One of us (R.A.P.) has found previously that injections of 10 μg of Cu^{2+} (as $CuCl_2$ in isotonic saline) inside the skull of pigeons under ether anaesthesia can give rise to convulsions and death: further studies have been carried out to determine whether this effect can be reversed by some –SH substances. As the effect of the Cu^{2+} could be reduced by holding the

birds during convulsions, experiments were done with initial injections of 20 μg Cu^{2+}. Five of six pigeons died with this injection alone; one, which was anaesthetized for 9 min after the injection, survived.

(1) *Protection experiments.* Penicillamine (dimethyl cysteine) holds up convulsions for 10–60 min and reduces their severity. Greater protection is given by penicillamine plus lipoate (–SH form), though BAL (dimercapto-propanol) did well in two experiments (Table 1).

TABLE 1. Effect of thiols on the toxicity of Cu^{2+} to the brains of pigeons

	Substances injected (μg)					
No. of animals	Cu^{2+}, 20–25	Penicill-amine, 100–500	Lipoate, 200	BAL, 600	No. died	Remarks
6	+	–	–	–	5/6	1 survival after deep anaesthesia
10	+	+	–	–	3/10	Convulsions after 20–60 min. 2 survived after deep anaesthesia
5	+	+	+	–	0/5	Minor symptoms at 10–21 min
5	+	–	+	–	1/5	Animal dying showed convulsions
2	+	–	–	+	0/2	One animal needed artificial respiration

(2) *Reversal experiments.* The next question was whether copper could be removed once it has combined with the tissues. The onset of convulsions was slowed by giving 20 μg Cu^{2+} together with 500 μg penicillamine (in one case copper plus 100 μg penicillamine). When convulsions appeared 12–30 min later the animal was again anaesthetized and a thiol injected. In no experiment were convulsions rapidly aborted. A further dose of penicillamine was ineffective. BAL alone, in one case, prolonged life for several hours; 200 μg lipoate (–SH form) plus BAL (600 μg) saved two birds, the other six all died. We conclude that some reversal can be obtained with mixtures of dithiols but this is not a regular effect.

Experiments *in vitro* suggest that the toxic action of copper in mitochondrial preparations is to block the entry of pyruvate into the citric acid cycle; it is possible that in the intact animal Cu^{2+} does not reach the mitochondria.

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Kinin-releasing enzyme in the accessory sex glands of the guinea-pig

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The accessory sex glands of the guinea-pig contain large amounts of a substance which releases a kinin from a substrate in its own plasma and from that of other mammals. The substrate is also present in lymph or serum globulin. This substance, probably an enzyme, is non-dialysable, heat labile, and is completely precipitated from aqueous solution by 80% acetone. The rate at which it releases the kinin is similar to the release of kallidin or bradykinin by kallikrein and trypsin. The release is unaffected, however, by incubation of the enzyme with soya bean trypsin inhibitor, ovomucoid trypsin inhibitor, or kallikrein inactivator (from ox parotid gland). The highest concentrations of enzyme are in the coagulating gland, about half this concentration is present in the prostate and one-fiftieth in the seminal vesicles. Freeze-dried coagulating or prostate gland was considerably more active than submaxillary gland. Detectable amounts of kinin could be released from plasma or serum globulin by as little as 5 μg freeze-dried coagulating gland. A kinin-releasing substance was not detected in aqueous extracts of freeze-dried testis, ovary, lymph gland, spleen, liver, gastric mucosa or thyroid gland of the guinea-pig.

The kinin released has been extracted from serum and plasma after incubation with the enzyme by the method for extracting kallidin and bradykinin (Holdstock, Mathias & Schachter, 1957). Parallel tests with bradykinin on the guinea-pig ileum, rat uterus and rat duodenum yielded ratios of activity which did not deviate significantly from unity; this kinin was, however, 3–4 times relatively more active than bradykinin on the arterial blood pressure of the rabbit. Like kallidin and bradykinin it was rapidly inactivated by plasma and chymotrypsin but not by trypsin. The coagulating gland and prostate gland also contain inactivators of this kinin and bradykinin.

The coagulating gland of the rat and the prostate gland of rat, rabbit, dog and man do not release a kinin from plasma. The presence of this enzyme in the prostate gland of only one of 5 mammals studied, its absence in gastric mucosa, liver and other glands of the guinea-pig, and the fact that the enzyme in guinea-pig saliva releases a kinin from plasma of other mammals but not from that of the guinea-pig (Schachter, 1960) do not support the suggestion (Hilton, 1960) that these enzymes afford a common mechanism whereby glandular tissues obtain the blood supply they need when actively secreting. It seems more likely, that in most instances, these enzymes in glands have an exocrine function and that their ability to release a kinin is incidental to this role.

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Pressor material in renal lymph

By A. F. Lever and W. S. Peart. *Medical Unit, St Mary's Hospital, London, W. 2*

Renal lymph has been collected by direct cannulation in the anaesthetized dog and examined for its pressor activity by either direct assay or indirect assay, after incubation with plasma, using the rat blood-pressure preparation (Peart, 1955, 1957–58). In all cases it has been found to contain pressor material. In the few cases where the presence of such material was initially uncertain, it could be readily identified after concentration by ultrafiltration. Lymph from various non-renal sources was examined both before and after concentration and in no case was pressor material found.

In eight experiments thoracic duct lymph was collected in the neck from the anaesthetized dog and pressor material identified in each by concentration and indirect assay. This activity disappeared after bilateral nephrectomy.

The pressor material in lymph resembled a standard preparation of dog renin in that both were heat labile and did not diffuse through a dialysis membrane. Both showed the same mobility characteristics on gel filtration columns of Sephadex (Pharmacia) capable of separating proteins from each other as well as from smaller molecules such as angiotensin. With an ion-exchange column of diethyl-amino-ethyl-cellulose (Peterson & Sober, 1956), adsorption and elution of both standard dog renin and the lymph activity occurred between the same ranges of buffer molarity and pH.

Equipressor amounts of renin and lymph were capable of forming a quicker acting pressor material on incubation with dog plasma containing renin substrate. The velocities of these reactions were comparable and the quick-acting product in each case resembled synthetic Val 5 angiotensin (Ciba) in its effect on the rat's blood pressure. This quick-acting material further resembled angiotensin in that it was heat stable, diffused through a cellophane membrane, had the same mobility on Sephadex and was destroyed by plasma containing high levels of angiotensinase activity.

In fifteen anaesthetized dogs a constriction was applied to one renal artery during the collection of lymph from both kidneys. Six showed a definite increase in the concentration of pressor material in lymph from the kidney with the stenosis. Five showed a questionable increase and four showed no increase. In no case did lymph from the control kidney become

more pressor than that from the stenosed side. In view of the possibility that these changes could represent an over-all concentration of renal lymph protein as a result of the stenosis, lymph nitrogen was measured in three experiments as an index of total lymph protein. In none of these did the nitrogen concentration differ at any time between the two sides, although in two definite increases in pressor material occurred in the lymph from the stenosed side.

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The structure and behaviour of transitional epithelium

By J. M. N. Boss. *Department of Physiology, University of Bristol*

The epithelium, termed transitional, which lines much of the urinary tract, becomes in the bladder greatly reduced in thickness when that organ is distended. Harvey (1909) suggested that, although the epithelial cells certainly become wider and flatter, they might also slip sideways over each other. Schaffer (1929) pointed out that it was unnecessary to suppose that the cells move upon each other, and some more recent authors have described the stretching of transitional epithelium entirely in terms of the deformation of the individual cells (Levi, 1946; Elias & Pauly, 1960). Nevertheless, Harvey's hypothesis is supported by current text-books, which state that, when the bladder is distended, the cells of the epithelium are displaced over one another to form fewer layers (Bremer & Weatherford, 1944; Kendall, 1947; Carleton & Short, 1956; Ham, 1950). Bloom (1957) writes that the number of cell layers appears to be reduced, but does not state whether the appearance is misleading. In view of this disagreement in the literature, the matter was re-investigated.

The bladders of four adult male white rats were fixed in an osmic-sublimate-dichromate mixture, one bladder being collapsed, one fully distended and two at intermediate stages of distension. Histological sections from each of the four blocks were stained with haematoxylin and eosin and by Masson's triple stain for general structure, with Heidenhain's iron haematoxylin and Mallory's phosphotungstic acid haematoxylin for cytoplasmic detail, and by the periodic acid-Schiff reaction for polysaccharides. From each block 4 sections stained by phosphotungstic acid haematoxylin and 14 by each of the other stains were examined. The granules already reported (e.g. by Bremer & Weatherford, 1944) in the most superficial cells, which are large and palely staining, were found to be coloured by the periodic acid-Schiff reaction.

The effects of distension on the vesical epithelium were as follows: (*a*) The thickness was reduced from 12–20 to 6–7 μ. (*b*) The rows of nuclei were reduced from 3 or 4 to 2 or even 1. (*c*) Examination of intercellular boundaries indicated great lateral deformation of all cells but did not suggest reduction in the numbers of layers of cells. From these results it is concluded that a reduction in thickness and in number of rows of nuclei need not imply any reduction in the number of layers of cells, and that it is unnecessary to assume that the cells of transitional epithelium slide over each other in the rat's bladder. On the other hand, the structure of the epithelium, as seen during this work, agrees well with Schaffer's description (1929).

This study was a class exercise in a Part I B.Sc. course. The following students participated: C. C. Ashley, J. M. Barker, Linda M. Blackmore, Robina Gyle-Thompson R. O. Law, Jennifer Lucas and J. M. Steadman.

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On the relation of the pulse to the ballistocardiogram

By I. Starr and S. Ogawa. *Department of Therapeutic Research, University of Pennsylvania, Philadelphia, Pa., U.S.A.*

Both pulse and ballistocardiogram have their origin in the energy of the heart's contraction and they must certainly be related, yet the ballistocardiogram often suggests abnormality of cardiac performance that is not apparent in inspection of the conventional pulse record. Results secured in several studies have suggested a possible explanation for the discrepancy. The theory of Noordergraaf & Heynekamp (1958) relates the 'force' ballistocardiogram to the 2nd derivative of the movement of the body's centre of gravity during the cardiac cycle; the pulse, the pressure change resulting from changes of volume of blood in any vessel, is related more directly to the movement itself. Also it was noticed by Reeves, Jones & Hefner (1957) that the calculated 2nd derivative of pulse curves often

looks rather like ballistocardiograms taken simultaneously; the same idea can be supported from results of cadaver experiments made in this laboratory (Starr, Horwitz, Mayock & Krumbhaar, 1950; Starr & Schnabel, 1954). From this evidence one cannot conclude that the 'force' ballistocardiogram *is* the 2nd derivative of the pulse, for this, mathematically speaking, is not true. But this evidence certainly suggests that the true relationship between the curve of pressure changes we call the pulse, and the 'force' ballistocardiogram, would be best expressed by some sort of differential equation. If this were true, one would expect that the information concerning cardiac abnormalities seen in the 'force' ballistocardiogram, but apparently absent from the pulse, would be made more obvious in the pulse if that record was differentiated. Accordingly, by means of a capacitance pulse transducer of the type designed by Brecht & Boucke (1953), connected with a differentiating electrical circuit, records of the 1st derivative of the brachial pulse have been secured on more than 200 persons, some with normal, some with abnormal hearts; while the ballistocardiogram was recorded simultaneously. Although there is difficulty calibrating the record of the pulse derivative, as there is in calibrating the conventional pulse record taken by a transducer pressing on the skin over an artery, the results show clearly that many abnormalities of myocardial function seen in the ballistocardiogram become obvious in the pulse also, when that record is differentiated.

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The effect of hypophysectomy on the gastric mucosa of the rat

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The parietal cell population of the whole stomach was estimated in rats, using the counting method described by Card & Marks (1960). Stomachs were counted 'blind'. 'Counts' were reproducible, and the errors in the sampling method were insignificant.

Hypophysectomized rats were compared with intact rats of similar body weight, but of younger age (see Table 1). The hypophysectomized rats were killed at a mean interval of 21 days after operation.

The parietal cell population was significantly lower in hypophysectomized as compared to 'control' animals ($P < 0.001$). This effect was entirely due to reduction in the volume of the gastric mucosa in the hypophysectomized group, parietal cell density (defined by the relationship 'total parietal count/mucosal volume') being unaltered. Preliminary evidence suggests that growth hormone (N.I.H.) can restore the mucosa of hypophysectomized animals to normal.

TABLE 1

Group	No.	Body weight ± S.D. (g)	Approx. age (days)
Hypophysectomized	11	95·5 ± 31·9 at operation 89·00 ± 19·8 when killed	38 59
'Normal'	10	103·2 ± 19·1	40

(The difference between the mean body weights of the normal and the hypophysectomized groups at time of killing was not significant, $P = 0.1$.)

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The propagation of vasodilatation in human skin by an apparently non-neural mechanism

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Radiant heating of the proximal part of the forearm causes a vasodilatation which extends to the distal, unheated part (Crockford & Hellon, 1959). Blood-flow determinations taken every 15 sec with strain-gauge plethysmographs showed that vasodilatation occurred in both parts of the arm at the same time. Crockford, Hellon & Parkhouse (1959) found that the centrifugal spread of the vasodilatation was not prevented by a subcutaneous ring of anaesthetic proximal to the heated area, but was blocked by a similar injection of anaesthetic in the mid forearm. Cervical sympathectomy had no effect on the vasodilator spread.

Experiments on three patients with complete brachial plexus lesions have now shown that the dilator spread still occurs, so that the mechanism does not appear to depend on any known fibre system. Indeed, the reaction is similar to the dilator response in the cat's femoral artery, which appears after tetanus of the leg muscles, and is apparently conducted along the smooth muscle of the artery wall (Hilton, 1959). We have shown that both this femoral artery mechanism in the cat, and the spread of the vaso-

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dilatation in the forearm, can be blocked by local application of adrenaline ($0{\cdot}5 \times 10^{-5}$).

A vasodilatation in the distal area of the forearm can also be produced by application of a rubefacient substance to the proximal part. The spread of this response could be blocked with a subcutaneous ring of lignocaine or adrenaline. Likewise ultra-violet radiation caused a simultaneous increase in blood flow in proximal and distal parts of the arm.

It is suggested that a wide variety of stimuli act to produce a local vasodilatation in the skin which spreads rapidly to neighbouring areas and is apparently conducted in the smooth muscle of the subcutaneous arterial plexus.

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Changes in the oxygen consumption of rabbits in response to blood-stream cooling

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Postural changes in gas distribution in the lungs and the effect of histamine inhalation in different postures

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The influence of posture on the functional residual capacity (F.R.C.) and on the degree of uneven gas distribution in the lungs has been studied in six healthy male subjects placed supine on a tilting table, in each of seven different positions, ranging from 50° head up to 50° head down. An open-circuit nitrogen wash-out method was used to measure F.R.C. and the 'lung clearance index' (L.C.I.), a measure of the unevenness of distribution of inspired gas in the lungs (Bouhuys & Lundin, 1959).

The results showed, for the F.R.C., a linear regression with the angle of tilt, amounting to an average decrease of the F.R.C. with 140 ml./10° of tilt from head up to head down. The L.C.I. increased significantly in the horizontal and head-down positions, compared to the values in the 20° and 50° head-up positions. This result indicates a more uneven gas distribution in the horizontal and head-down positions than in the head-up positions, and this may be explained by the decrease of the F.R.C. in the former positions.

According to Clements, Brown & Johnson (1958), bronchial muscle tone and surface forces in alveoli and small airways are the main factors which determine participation of lung units in ventilation when the lungs are deflated. In our subjects a comparison of the nitrogen wash-out in the 30° head-down position before and after epinephrine inhalation showed no significant change in the values of F.R.C. and L.C.I. Therefore, mechanical factors acting at moderate and small lung volumes, rather than changes in bronchial muscle tone, are probably the main cause of the observed changes in gas distribution.

The L.C.I. has been shown to be a sensitive measure of the bronchoconstrictive effect of inhaled histamine (Bouhuys, Jönsson, Lichtneckert, Lindell, Lundgren, Lundin & Ringquist, 1960). In the present study five subjects inhaled a histamine aerosol in the 50° head-up and in the 20° head-down positions. In each subject the effect of the drug (at the same concentration in the nebulized fluid) on the L.C.I. was more marked in the head-down than in the head-up position. This result, which is probably caused by a decrease of the average bronchial diameter in the head-down position, may explain, in part, why asthmatic patients often develop attacks of bronchoconstriction when lying in bed.

The authors thank Mr P. D. Oldham (Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glamorgan), who performed the statistical analysis.

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Recovery from adaptation in the rabbit lung-thorax

By L. Bernstein, M. P. Godfrey and Mary C. Riley. *Physiology Research Laboratory, Veterans Administration Hospital, and Department of Environmental Medicine, School of Hygiene, Johns Hopkins University, Baltimore, Maryland, U.S.A.*

Pressure–volume curves of the lung–thorax of live, anaesthetized, curarized rabbits of about 2 kg body wt. were determined by Bernstein's (1957) method, in the volume range from functional residual capacity (F.R.C.) to approximately total capacity (T.C.). Reference curves were obtained in the following conditions: (1) in the first inflation beyond tidal volume, (2) in deflation from T.C., and (3) in early re-inflation to T.C. In subsequent experiments the lungs were inflated to T.C., partially deflated to some volume in the range down to F.R.C., and finally re-inflated stepwise to T.C.

If a decrease in incremental compliance at any pressure or volume is due to a reduction in the number of functioning 'alveoli', collapse of alveoli during deflation should cause the area beneath the pressure–volume curve to be greater in re-inflation than in deflation (pressure plotted as ordinate). When deflation was to a level 10 c.c. or more above F.R.C., re-inflation either immediately, after 1 min in the deflated state, or after 1 min of tidal ventilation between the chosen level of deflation and 5–15 c.c. above it, all yielded essentially similar pressure–volume curves, with no indication of alveolar collapse. When deflation was to within 10 c.c. of F.R.C., the curves for both immediate and simple, delayed re-inflation indicated a small amount of collapse, but the curve for re-inflation after tidal ventilation indicated a much greater amount.

We conclude that: (1) alveoli have critical closing pressures (C.C.P.) as well as critical opening pressures (C.O.P.), and (2) closure or collapse occurs during the process of deflation, not while the alveolus is in the deflated state—if an alveolus has not collapsed by the time the lung–thorax has deflated to the chosen volume, it is unlikely to do so however long the lung–thorax remains in that state. A possible explanation is that during deflation the most rapidly emptying units close when their transmural pressure falls below their C.C.P., while other, slower emptying units still have transmural pressures above their C.C.P. Some of the gas expelled from the units collapsing enters the more slowly deflating units, limiting the extent of their deflation; these units are so prevented from collapsing. On Clements, Brown & Johnson's (1958) 'surface tension' hypothesis, C.O.P.s and C.C.P.s of individual units should be positively correlated, C.O.P. being the larger. Collapsed units would therefore be unlikely to reopen in the next, small, tidal inflation, but the next tidal deflation would afford a second opportunity for the now fastest emptying group of alveoli to collapse at the expense of the remainder. Thus, each tidal ventilation would increase the number of units collapsed and insusceptible of re-inflation without exceeding their C.O.P.—i.e. would cause a gradual reduction of compliance.

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The measurement of an unconventional lung volume in rats

By H. D. Singh and O. L. Wade. *Department of Therapeutics, Queen's University, Belfast*

A method based on the constancy of the product of pressure and volume (Boyle's Law) is used to measure small gas volumes in a fluid-filled rigid system (Fig. 1).

Rats are killed by chloroform inhalation. A ligature is tied around the neck and the rat is placed in the Kilner jar. The volume of water that has

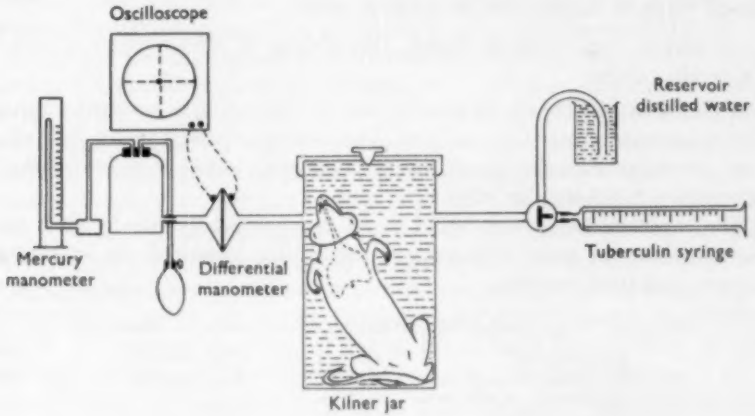

Fig. 1

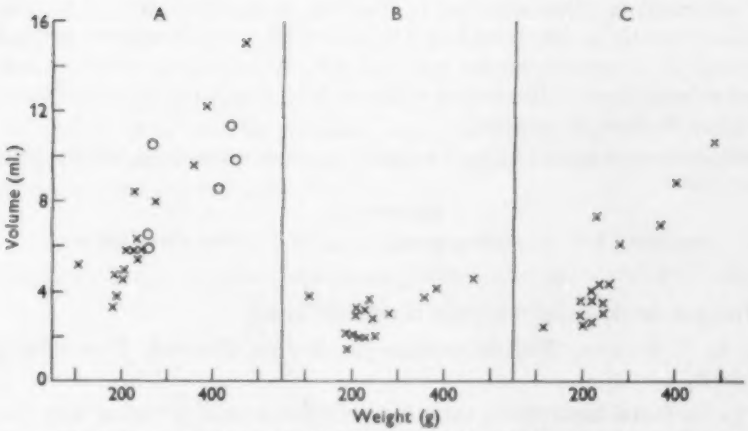

Fig. 2. A, gas in whole animal; B, gas in lung-free body; C, lung volume.

to be added to the system to raise the pressure to 100 mm Hg is measured. The measurement is repeated after removal of lungs of the animal.

The relation between lung volume and body weight of 16 normal albino rats (I.C.I. strain) is shown in Fig. 2C. The coefficient of linear correlation $r = 0{\cdot}895$. Regression formula: lung volume in ml. = 0·025 weight in grams − 1·43 *or* = 1·12.

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Blood oxygen saturation in normal rats

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A cuff was inflated to 80 mm Hg around the tail of a warmed normal albino laboratory rat (I.C.I. strain). Samples of capillary blood, 0·04 ml., were obtained from the tip of the tail and analysed by a micro method (Roughton & Scholander, 1934).

The capillary blood was found to be desaturated (Table 1) when the animals were at rest. The oxygen saturation increased on breathing oxygen and after exercise.

Table 1. Oxygen content of capillary blood samples in 10 normal rats

Breathing air	18·6 vol. %	(S.D. = 0·57)
Breathing oxygen	21·1 vol. %	(S.D. = 1·15)
Significance of difference	$0{\cdot}001 > P$	
Oxygen capacity	21·6 vol. %	(S.D. = 1·10)

Various methods have been used to confirm that the oxygen saturation of tail capillary blood sampled in this way is representative of arterial blood. Analysis of samples of blood taken by left ventricular puncture and by indwelling carotid canulae and oximetry of shaved skin folds confirm that arterial blood of the resting rat is not fully saturated. The significance of these findings is discussed.

We acknowledge grants from the Northern Ireland Hospitals Authority and The Wellcome Trust.

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Changes in O_2 supply within the foetal lamb

By G. S. Dawes. *Nuffield Institute for Medical Research, University of Oxford*

In the foetal lamb the O_2 saturation of carotid blood is higher than that in the femoral or umbilical arteries; the difference is small and rarely

exceeds 10%. Yet Barcroft (1946, p. 202) found differences of 29–59% in four out of five lambs about half way through gestation. The discrepancy could only be resolved in part by the fact that Barcroft's samples were not withdrawn simultaneously, and a search was made for other causes. Among the possibilities considered was spasm of the umbilical veins, due to handling or puncture of the vessels, with reduction of umbilical blood flow and a fall in the proportion of oxygenated blood reaching the right side of the heart.

The abdominal part of the umbilical vein was exposed in foetal lambs under general anaesthesia, and was partly constricted with a tape. In lambs of 80–86 days' gestation age (term is 147 days) this caused a fall in both carotid and femoral arterial O_2 saturations, but the latter fell further, so that the carotid–femoral O_2 difference was more than doubled. Hypoxia alone, induced by giving the mother low O_2 mixtures, caused a decrease in the carotid–femoral arterial O_2 difference because umbilical venous O_2 saturation was reduced. With an electromagnetic flowmeter in the umbilical vein, flow had to be reduced by 50% or more (usually to less than 100 ml./kg. min) to cause an increase in the carotid–femoral arterial O_2 difference; this was accompanied by a fall in foetal O_2 consumption. Partial obstruction of umbilical venous flow caused a large rise in umbilical vein pressure, and a fall in blood pressure and pulse pressure, suggesting that a substantial quantity of foetal blood had accumulated in the placenta, which is about the same weight as the foetus at this age.

In contrast, in mature foetal lambs (134–145 days' gestation) whose weight is at least five times greater than that of their placenta, progressive reduction of umbilical blood flow by venous constriction caused no increase in carotid–femoral arterial O_2 difference, but haemorrhage (30 ml./kg or more, sufficient to reduce flow to less than 100 ml./kg. min) did cause an increase. This suggests that it is reduction of foetal blood volume, combined with a fall in umbilical flow, which is of crucial importance.

Although it is possible to cause large changes in the distribution of oxygenated blood within the foetal circulation, this entails large deviations from a normal physiological condition.

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The site of action of nerves in the pulmonary vascular bed of the dog

By I. DE BURGH DALY and B. A. WAALER.* *University Laboratory of Physiology, Oxford*

In the dog the predominant effect on the pulmonary vascular bed of stimulating the nerve fibres from the upper thoracic sympathetic outflow is vasoconstriction. The fibres concerned relay in the stellate or middle cervical ganglion and proceed to the lungs in the thoracic vagosympathetic nerve trunk (Daly, Duke, Hebb & Weatherall, 1948). The vasoconstrictor response can be demonstrated in isolated innervated perfused lungs in the absence of passive bronchomotor effects. The site of action in the pulmonary vascular bed of the nerve fibres concerned is not known. An attempt to locate this site has been made in experiments with isolated lung lobe preparations of a type previously described (Daly & Waaler, 1960). The pulmonary circulation perfusion was temporarily stopped and the intratracheal pressure raised in order to compress the capillaries. Tests showed that the intratracheal pressure applied (20–25 mm Hg) was sufficient to prevent the transfer of blood from arteries to veins or vice versa. Sensitive volume recorders were put in connexion with the pulmonary arterial cannula and with either a cannula inserted into the quiescent left atrium or a cannula inserted into a lobar vein.

The preparations were atropinized in order to eliminate bronchomotor responses with their possible passive effects on the pulmonary vascular bed. Bronchial circulation perfusion was maintained throughout the experiments, but was temporarily interrupted during some of the nerve stimulation tests.

Under these conditions electrical stimulation of the thoracic vagosympathetic nerve caused a diminution in volume of both the arterial and the venous side of the pulmonary vascular bed in seven out of eight experiments. The remaining experiment showed a reduction in volume on the arterial side but not on the venous side. In one preparation the diminution in volume on the arterial side was preceded by a short-lasting increase in volume. When calculated for both lungs the volume reductions caused by the stimulations ranged from 2·8 to 7·5 ml. on the arterial side and from 0 to 2·2 ml. on the venous side, the mean values being 4·1 and 1·0 ml. respectively. (The weight of the dogs ranged from 22 to 30 kg; average 25 kg.)

In about half the nerve stimulation tests the arterial and venous capacity responses started almost simultaneously. In the remaining tests either the arterial or the venous response preceded the other by a significant time interval.

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The results are interpreted as indicating that sympathetic vasoconstrictor fibres act on the arterial as well as on the venous side of the pulmonary capillaries.

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Effects produced by distension of the pulmonary artery

By J. C. G. COLERIDGE and C. KIDD. *Department of Physiology, School of Medicine, University of Leeds*

The reflex effects which can be elicited from the pulmonary circulation have been examined by several workers (reviewed by Heymans & Neil, 1958). In continuation of experiments which have shown the distribution, nerve connexions and threshold of baroreceptors in the extrapulmonary parts of the pulmonary artery of the dog (Coleridge & Kidd, 1960*a*, *b*; Coleridge, Kidd & Sharp, 1961) a further attempt has been made to investigate the possible role of this vessel as a reflexogenic area.

In anaesthetized dogs with the chest open the right lung roots were ligated. The right pulmonary artery between its origin and the lung root was then converted into a closed sac by inserting a tube through an incision in the main pulmonary trunk and tying it in position; one end of the tube lay in the origin of the pulmonary trunk, the other in the left pulmonary artery. Only the left lung was ventilated and it received the whole output of the right ventricle through the tube. The isolated sac, which retained its nerve supply intact, could then be perfused with blood through the lobar arteries.

Pulsatile distension of the isolated right pulmonary artery, with pressures of 20–60 mm Hg, produced either no effect or a fall in systemic arterial pressure of up to 55 mm Hg accompanied in some cases by a brief and variable slowing of the heart rate. The effects were abolished by cooling or section of the cervical vagus nerves. Recordings of afferent vagal impulse activity showed increased discharge from baroreceptors in the isolated right pulmonary artery during distension.

By contrast, distension with abnormally high pressures (e.g. greater than 90 mm Hg) produced a rise of up to 80 mm Hg in systemic arterial pressure, accompanied by respiratory movements of the chest wall and diaphragm. Action potential studies suggested that these effects, which were abolished by vagal section, were due to activation of chemoreceptors in the vicinity of the pulmonary artery. It is suggested that the blood supply of these chemoreceptors was occluded by the abnormally distended right pulmonary artery.

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Chronic effect on blood pressure of combined bilateral carotid and vertebral ligation in the rabbit

By R. D. LOWE. *Medical Unit, University College Hospital Medical School, London, W.C.* 1

Rosenfeld (1952) and Rothman (1955) have reported chronic hypertension following bilateral carotid ligation, but their method of blood-pressure recording is open to criticism. Wakerlin, Crandall, Frank, Johnson, Pomper & Schmid (1954) and Crandall, McCrorey, Sukowski & Wakerlin (1957) have reported hypertension following carotid stenosis in the dog, but interfered with buffer nerve function in their operative procedure. In man, speculation has ranged from the view that carotid stenosis might be an occasional cause of hypertension to the suggestion that carotid and vertebral narrowing might be the cause of essential hypertension (Dickinson & Thomson, 1960).

The effects of bilateral carotid or vertebral ligation have been studied in the rabbit using the central artery of the ear for recording blood pressure (a method of carotid ligation was used which preserves this artery). These animals did not develop hypertension or any lasting cerebral ischaemia, because the posterior communicating artery dilates markedly.

A further series of 26 rabbits was prepared using combined bilateral carotid and vertebral ligation. Over a 5-week period both internal carotid arteries, both external carotids, one vertebral and the ascending cervical artery were cut, and the remaining vertebral artery was constricted. Sixteen rabbits survived these procedures and in twelve of them, 5–10 weeks later, the stenosed vertebral and the remaining ascending cervical artery were cut. The ten survivors were shown to have a markedly lowered retinal artery pressure, but no animal became hypertensive at any stage during the six months period of study.

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The influence of phenoxybenzamine on the uptake of noradrenaline by cat and rat tissues

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The uptake of noradrenaline by sympathetically innervated tissues may contribute to the disappearance of circulating noradrenaline (Axelrod, Whitby & Hertting, 1960; Pennefather & Rand, 1960). As the adrenergic blocking agent phenoxybenzamine increases the concentration of noradrenaline in the splenic venous effluent when the sympathetic postganglionic fibres are stimulated (Brown & Gillespie, 1957), it was of interest to study the effect of phenoxybenzamine on noradrenaline uptake by the tissues.

Phenoxybenzamine (10 mg/kg) was given intravenously to non-pregnant spinal cats, and one kidney and one uterine horn were removed 15 min later. Noradrenaline (1 mg) was then infused at a rate of 25 μg/min, and 20 min after the infusion the other kidney and uterine horn were removed. The tissues were extracted and assayed on the blood pressure of the pithed rat. Noradrenaline uptake by these tissues was measured by the method of Pennefather & Rand (1960).

Phenoxybenzamine prevented the noradrenaline uptake by the kidney, a tissue in which its excitatory action is blocked by phenoxybenzamine. In contrast, it did not prevent the noradrenaline uptake by the uterus, a tissue in which the inhibitory action of noradrenaline is unaffected by phenoxybenzamine. There may be therefore a relationship between the adrenergic blocking property of phenoxybenzamine and its effect on the noradrenaline uptake. This view is supported by the finding that dichloro-*iso*-prenaline (10 mg/kg), an antagonist of adrenergic inhibitory responses, prevented the noradrenaline uptake by the uterus but not that by the kidney.

We then examined the effect of phenoxybenzamine (6 mg/kg) on the noradrenaline uptake by tissues of pithed rats. The uptake was marked in the heart but much less so in the spleen, uterus and duodenum after infusions of 80 μg over 20 min; this marked uptake by the heart was reduced by phenoxybenzamine. The single dose of phenoxybenzamine *per se* depleted the spleen of noradrenaline but did not alter the noradrenaline levels in the heart, uterus or duodenum. Prolonged treatment with phenoxybenzamine (6 mg/kg for 7 days) lowered the noradrenaline

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content of the heart as well as that of the spleen (as found by Schapiro, 1958), but such treatment had no effect on that of the uterus or duodenum.

Thus, phenoxybenzamine produces depletion of noradrenaline, and prevents the uptake of noradrenaline, in tissues where the action of this catecholamine is excitatory. We suggest that the adrenergic blocking agent interferes with the binding of noradrenaline in these tissues.

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